Diversity and Host Interaction of Phthorimaea operculella granulovirus

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“Look deep into nature and you will understand everything better.”
- Albert Einstein -

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List of Abbreviations

%	percent
#	number
⌀	diameter
× g	multiple of gravitational force
°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
aa	amino acid
ABC	Andermatt Biocontrol AG, Grossdietwil, Switzerland
bp	base pair
BSA	bovine serum albumin
Bt	*Bacillus thuringiensis*
BV	budded virion(s)
CIP	International Potato Center
cm	centimetre
CNI	Close-Neighbor-Interchange
Da	dalton
df	degrees of freedom
DNA	desoxyribonucleic acid
ddH_2O	bidistilled water
dH_2O	distilled water
dNTP	desoxynucleoside triphosphate
DP	read depth
DP4	number of reads covering reference forward, reference reverse, alternative forward and alternative reverse
dpi	days post infection
DPR	read depth of different alleles for reference, alternative 1, alternative 2 and alternative 3
dsDNA	double stranded desoxyribonucleic acid
rDNA	DNA sequence that codes for ribosomal RNA
e.g.	*exempli gratia*; for example
egt	ecdysteroid UDP–glucosyltransferase
EPPO	European and Mediterranean Plant Protection Organization
et al.	*et alii*; and others
g	gram
GQ	genotype quality
gran	granulin
GV	granulovirus
h	hour
i.e.	*id est*; that is
IGS	intergenic spacer
Indel	insertion or deletion
ITS	internal transcribed spacer
JKI	Julius Kühn-Institut

K-2-P	Kimura 2-parameter
kbp	kilobase pair
kDa	kilo Dalton
kg	kilogram
L1 - L4	larval stage
l	liter
LC_{50}	median lethal concentration
LEF	late expression factor
log	logarithm
LT_{50}	median lethal time
LSU	large subunit rRNA gene
m	meter
ME	minimum evolution
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MNPV	multiple nucleopolyhedrovirus
n	number of tested individuals
N	number of independent replicates
n.d.	not determined
ng	nanogram
NGS	next generation sequencing
nm	nanometer
NNI	Nearest-Neighbor-Interchange
no.	number
NPV	nucleopolyhedrovirus
nt	nucleotide
OB	occlusion body(-ies)
ODV	occlusion derived virion(s)
ORF	open reading frame
polh	polyhedrin
PCR	polymerase chain reaction
Phop	Phthorimaea operculella
Phop-EG	Phthorimaea operculella population from Egypt
Phop-IT	Phthorimaea operculella population from Italy
Phop-TN	Phthorimaea operculella population from Tunisia
PhopGV	Phthorimaea operculella granulovirus
pH	-log10 (aH^+)
pM	picomolar
PTM	potato tuber moth
QUAL	Phred-scaled probability of all samples being homozygous to reference
rDNA	ribosomal desoxyribonucleic acid
REN	restriction endonuclease
RFLP	restriction fragment length polymorphism
RPB1	RNA polymerase II largest subunit
RNA	ribonucleic acid
RT	room temperature
SD	standard deviation

SDS	sodium dodecyl sulfate
SE	standard error
sec	seconds
SNP	single nucleotide polymorpphism
SNPV	single nucleopolyhedrovirus
SOD	superoxide dismutase
sp.	species
spp.	*species pluralis*; multiple species
ssrDNA	small subunit ribosomal DNA
SSU	small subunit rRNA gene
TAE	Tris base, acetic acid and EDTA buffer solution
Taq	DNA polymerase from *Thermus aquaticus*
TE	Tris base and EDTA buffer solution
TEM	Transmission electron microscope
Tris	tris(hydroxymethyl)aminomethane
U	enzyme unit
UPNA	Public University of Navarra
UV	ultraviolet light
V	volt
v/v	volume per volume
w/v	weight per volume

Summary

Phthorimaea operculella granulovirus (PhopGV, *Baculoviridae*) has the potential to serve as biological control agent against *Phthorimaea operculella* (Zeller) and *Tecia solanivora* (Povolny) in the potato production cycle and *Tuta absoluta* (Meyrick) in tomato production under greenhouse conditions. These three pest insect species are closely related and belong all to the Gelechiidae family of Lepidoptera. Due to the climate change and global trade these pest insects have the potential to spread worldwide. PhopGV has already successfully been used as biocontrol agent in Latin America and North Africa mainly against *P. operculella* in potato field and storehouses. Research on the diversity of PhopGV isolates and their interaction with the host is strongly needed, to gain knowledge which allows optimizing the use of PhopGV as active ingredient of biocontrol agents of these pest insects in food production.

This study focused on PhopGV isolates and *P. operculella* as host system. It embraces the interaction of PhopGV isolates in case of co-infections and the interaction of PhopGV and a microsporidium when infecting the same host individual. A number of nine PhopGV isolates were tested on their biological activity against *P. operculella*. Median lethal concentration (LC_{50}) and median lethal time (LT_{50}) were determined as comparable measures of isolates´ virulence. PhopGV is a slow-killing virus which is able to inhibit pupation of infected host species. Virulence of different PhopGV isolates seems to be not only virus but also host dependent.

Twelve complete genome sequences of PhopGV isolates from passages of virus isolates collected from four different continents (Africa, South America, Asia and Europe) were analysed after Illumina Next Generation Sequencing (NGS). These geographic isolates of PhopGV are genetically highly similar but were rarely genetically homogeneous and appeared in most cases as mixtures of multiple genotypes. A new grouping system (1-4) could be developed based on single nucleotide polymorphisms (SNPs) as well as insertions and deletions (Indels) spread over the PhopGV genome. Further, a highly variable gene of the superoxide dismutase (*sod,* ORF 54) was identified. Previously, only variability of ecdysteroid UDP–glucosyltransferase (*egt,* ORF 129) alone was used as a grouping system for PhopGV isolates.

Virus infections of insects can easily stay undetected, without showing typically signs of a disease and do not need to be lethal. A virus named PhopGV-R could be isolated from a laboratory population of *P. operculella*. Crowding of larvae did not cause overt outbreak of the covert virus. An infection with a second homologue virus (PhopGV-CR3) activated the internal virus. Whereas a third isolate, namely PhopGV-GR1, was able to suppress the internal virus and showed superinfection exclusion.

This research shows that stable virus infections seem to be common for insect populations and have an impact on population dynamics. It revealed that PhopGV isolates can either tolerate or block each other.

A potentially new *Nosema* species (*Nosema* sp. Phop) was purified from microsporidian infected individuals of *P. operculella.* It was found that an infection of *P. operculella* larvae with *Nosema* sp. can reduce PhopGV-caused mortality and thus showed an antagonistic effect against PhopGV. These findings of virus-virus and virus-microsporidium interaction can help to predict the mode of action if PhopGV is applied against *P. operculella* field populations, where other PhopGV isolates or microsporidia can naturally occur.

Zusammenfassung

Phthorimaea operculella Granulovirus (PhopGV, *Baculoviridae*) ist ein potentieller biologischer Wirkstoff zur Bekämpfung der Kartoffelmotte, *Phthorimaea operculella* (Zeller) und der Guatemaltekischen Kartoffelmotte, *Tecia solanivora* (Povolny) in der Kartoffelproduktion sowie der Tomatenminiermotte, *Tuta absoluta* (Meyrick) im Gewächshaus bei der Tomatenproduktion. Diese drei genannten Schadinsektenarten gehören alle der Familie Gelechiidae (Lepidoptera) an. PhopGV wurde bereits erfolgreich als biologisches Pflanzenschutzmittel in Lateinamerika und Nordafrika, gegen *P. operculella* im Feld bzw. Kartoffellager, eingesetzt. Forschung auf dem Gebiet biologischer Bekämpfungsstrategien gegenüber Gelechiidae Arten im Nutzpflanzenanbau ist von sehr großer Bedeutung, da durch Klimaerwärmung und globalen Handel mit Lebensmitteln die weltweite Ausbreitung dieser Schadinsekten begünstigt wird. Daher fällt ein Fokus auf die Diversität natürlich vorkommender Baculovirus Isolate, wie im vorliegenden Fall PhopGV und dessen Interaktion mit seinem Wirt. Der durch diese Forschung erlangte Wissensgewinn kann letztlich deutlich zur Optimiertung des Einsatzes von PhopGV als aktiven Wirkstoff im biologischen Pflanzenschutz dienen. Das angestrebte Ziel, den schädlichen Einfluss von Gelechiidae Arten auf die Lebensmittelproduktion zu vermindern, würde dadurch in greifbare Nähe rücken.

Zur Erstellung einer soliden Datenlage für die vorliegende Dissertation wurden zunächst neun PhopGV Isolate auf ihre biologische Aktivität gegenüber *P. operculella* untersucht. Als Vergleichswerte wurden hierzu die mittlere letale Konzentration (LC_{50}) und die mittlere letale Zeit (LT_{50}) bestimmt. Unter Berücksichtigung, dass es sich bei PhopGV um ein „slow-killing“ Virus handelt, was bedeutet, dass die Mortalität im Vergleich zu anderen Baculoviren erst spät eintritt, wurde die Verpuppungsrate als Parameter in Betracht gezogen. Es zeigte sich, dass eine PhopGV Infektion eine Verpuppung des Wirts verhinderte. Mit anderen Worten werden infizierte Larven zwar nicht sofort durch die Infektion durch das Virus getötet, die dadurch vermittelte Unfähigkeit zur Verpuppung verhindert allerdings das Erreichen des adulten Entwicklungsstadiums und somit auch zuverlässig die Fortpflanzung. PhopGV hat dadurch das Potenzial Wirtspopulationen dauerhaft zu minimieren. Eine weitere Erkenntnis der durchgeführten Biotests war, dass die Virulenz verschiedener PhopGV Isolate nicht nur virus- sondern auch wirtsabhängig zu sein scheint. Mit anderen Worten reagiert eine Wirtspopulation unterschiedlich auf verschiedene Virus Isolate, aber auch ein einzelnes Isolat erzielt unterschiedliche Mortalität, wenn es gegen eine alternative Wirtspopulation eingesetzt wird.

Auf Grundlage der unterschiedlichen Ergebnisse der Biotests wurden zwölf PhopGV Isolate ausgewählt, welche Passagen von Isolaten von vier verschiedenen Kontinenten (Afrika, Südamerika, Asien und Europa) waren und per „Illumina Next Generation Sequencing (NGS)“ sequenziert sowie die erhaltenen Daten analysiert. Dabei hat sich gezeigt, dass Isolate ungeachtet der geographischen Herkunft genetisch betrachtet sehr ähnlich, allerdings selten genetisch homogen sind und in den meisten Fällen als Mischung multipler Genotypen auftreten. Auf Grundlage der Sequenzierungsdaten konnte ein neues Gruppierungssystem (1-4) für PhopGV Isolate etabliert werden. Diese Gruppierung legt Polymorphismen, die über das gesamte Virusgenom vorliegen zu Grunde. Des Weiteren war es möglich mit *sod* (ORF 54) ein variables Gen zu identifizieren. Bisher wurde eine Gruppierung verschiedener PhopGV Isolate lediglich auf Basis eines einzigen variablen Gens namens Ecdysteroid UDP–Glucosyltransferase (*egt,* ORF 129) durchgeführt.

Innerhalb von Insektenpopulationen können dauerhafte, stabile Virusinfektionen leicht unentdeckt bleiben, wenn typische Zeichen einer Erkrankung ausbleiben und der Krankheitsverlauf einen subletalen Charakter aufweist. Ein solches Virus mit dem Namen PhopGV-R konnte von einer *P. operculella* Laborpopulation isoliert werden. Überbevölkerung, das sogenannte „crowding“ von Larven, führte nicht zum sichtbaren Ausbruch dieses Virus. Doch eine Sekundärinfektion mit einem homologen Virus (PhopGV-CR3) aktivierte das populationsinterne Virus und führte zu einer offen sichtbaren Infektion. Wohingegen ein drittes Virus Isolat mit Namen PhopGV-GR1, das interne Virus blocken konnte und einen Ausschluss der co-Infektion, die sogenannte „superinfection exclusion“, zeigte. Diese Forschung zeigt, dass stabile Virusinfektionen von Insektenpopulationen offenbar nicht die Ausnahme, sondern die Regel darstellen. Die Folge stabiler Virusinfektionen ist ein Einfluss auf die Populationsdynamik. Co-Infektionen von populationsinternen Virus Isolaten mit zusätzlich eingebrachten Virus Isolaten werden in manchen Fällen erlaubt, hingegen in anderen Fällen verhindert.

Abschließend konnte ein potentiell neues Mikrosporidium (*Nosema* sp. Phop) aus *P. operculella* isoliert und beschrieben werden. Dieses Mikrosporidium zeigte einen antagonistischen Effekt gegenüber PhopGV in *P. operculella* Larven. Die Erkenntnisse von Virus-Virus sowie Virus-Mikrosporidium Interaktionen können dabei helfen die Wirkweise von PhopGV vorherzusagen, wenn es im Feld gegen *P. operculella* zum Einsatz kommt, vor dem Hintergrund, dass dort andere PhopGV Isolate oder Mikrosporidien natürlicherweise vorkommen können.

Chapter I

Introduction

Gelechiidae and Their Impact on Global Food Production

Pest insects of the Gelechiidae family cause damage in Solanaceae crops worldwide and lead to serious economic loss (Desneux et al., 2010; Espinel-Correal et al., 2010; Kroschel and Koch, 1994; Roux et al., 1992). Three major Gelechiidae pest insects in crop production and food storage are the potato tuber moth (PTM) *Phthorimaea operculella* (Zeller), Guatemalan potato moth *Tecia solanivora* (Povolny) and tomato leafminer *Tuta absoluta* (Meyrick) which are responsible for high economical damage on crop plants.

P. operculella is reported to occur in more than 90 countries worldwide, in almost all tropical and subtropical potato production systems in Africa, Asia and Central and South America (Kroschel and Sporleder, 2006). Host plants are Solanaceae, like potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* L.), capsicum (*Capsicum anuum* L.), eggplant (*Solanum melogena* L.) and tobacco (*Nicotiana tabacum* L.) (Lacey and Kroschel, 2009). Significant economic loss may also occur in more temperate climates, such as Southern Europe and the Pacific Northwest of the United States though cold winters there generally restrict its development and reduce its status as a pest (Sporleder et al., 2004; Sporleder et al., 2008). The ongoing process of global warming could facilitate the distribution of PTM to temperate climate zones in the future. Crop loss of staple foods like potato is caused by infestation by insect larvae which feed and mine on potato tubers. Damage from insect feeding on crops further opens entry to secondary infections with plant pathogenic bacteria or fungi (Agriotos, 2005).

T. absoluta is a serious problem for tomato production in South America, described first in Peru 1917 and then in Chile, Brazil, Bolivia, Colombia, Ecuador, Uruguay, Venezuela and Argentina (Gómez Valderrama et al., 2017), and similar to PTM, the cosmopolitan spread of *T. absoluta* is going on rapidly. *T. absoluta* was reported in Spain in 2006 and soon emerged to South Europe, North Africa, the Mediterranean basin and Asia (Urbaneja et al., 2012; Desneux et al., 2010). It is reported to be one of the most serious pests in tomato production in the Mediterranean area, Middle East, Eastern, Central and Western Europe and North Africa (Allache et al., 2015). In the Northern hemisphere, *T. absoluta* became an important pest especially in greenhouse production of tomato, where natural climatic conditions are not relevant for its distribution (Gómes Valderrama, 2017; Allache et al., 2015).

T. solanivora is a major pest in potato storage rooms in Central, North and South America where it can damage up to 100% of the stored potato tubers (Vargas, 2004). The putative origin of *T. solanivora* is Guatemala where its common name Guatemalan potato moth derived from (Povolny, 1973). Since 1999, *T. solanivora* has been present on the Canary Islands; its spread to the European continent was aimed to be avoided with EPPO quarantine measures but today it can be found also on Spain mainland (EPPO, 2005).

Potential Pest Control Approaches against Gelechiidae

To control these pest insects chemical insecticides have been prevalently used. Resistance to chemicals was reported to have developed rapidly for all of these three pest species (Bacca et al., 2017; El-Kady, 2011; Dogramaci and Tingey, 2008; Lietti et al., 2005; Kay and Collins, 1987; Haines, 1977). In addition to the development of resistance to chemicals, considerable health risks for growers applying chemical pesticides have been reported (Crissmann et al., 1998). Alternative methods used for insect pest control are the application of biological control agents, *e.g.* beneficial insects (parasitoids, predators) or pathogens (Lacey and Kroschel, 2009). The release of parasitoids of the family Braconidae and Encyrtidae lead to a successful establishment of natural enemies of PTM in some countries, *e.g.* in Zimbabwe the occurrence of PTM was reduced that it was eventually eliminated as a significant potato pest (Mitchell, 1978). However, classical biological control with introduction of exotic antagonists for establishment and long-term control in those regions, where an insect pest has been unintentionally introduced and not effectively controlled by native natural enemies, has its limitations (DeBach, 1964). Introduced beneficial insects need to deal with the varying conditions of different climatic zones and to integrate in an existing ecological system. Many insects did not establish after introduction or did not function on a level which allows a successive control of a pest insect (Lacey and Kroschel, 2009). Also entomopathogenic nematodes of the genus *Steinernema* (Koppenhöfer, 2007; Kaya and Gaugler, 1993) and fungi like *Metarhizim anisopliae* or *Beauveria bassiana* (Sewify et al., 2000; Hafez et al., 1997) or botanicals like water extracts of *Azadirachta indica* A. Juss. (Kroschel, 1996; Salama, 2000) have been tested against PTM. *Bacillus thuringiensis* (Bt) is the only bacterium evaluated for PTM control. Biopesticides based on Bt are widely used microbial pesticides and are commercially produced for use against a broad range of lepidopteran pests including PTM (Kroschel and Koch, 1996). Because of rapidly developing resistances and hazardousness of chemicals and the sometimes failing applicability of biological control measures, more biological alternatives are needed, to widen the spectrum of control methods. Baculoviruses appear as powerful active ingredients for biocontrol products for many lepidopteran pests (Haase et al., 2015).

Baculoviruses in Plant Protection

First attempts for the control of pest insects in the forest occurred in 1892, with the introduction of virus diseased *Lymantria monacha* larvae into populations of the nun moth in pine forests in Germany (Huber, 1986). Field trials with baculoviruses were performed in the United States in 1913 (Cunningham, 1995). Baculoviruses have the potential to cause the collapse of insect populations like the Gilpinia hercyniae nucleopolyhedrovirus (GiheNPV) of the spruce sawfly, *Gilpinia hercyniae*, which was accidentally introduced to Canada in the 1930s (Cunningham, 1995). This GiheNPV was propagated and applied in selected locations for population control of *Gilpinia hercyniae* (Balch and Bird, 1944). Several baculovirus-based products came to registration in Canada between 1983 and 2006 and were used as biological control agents against eruptive forest defoliators. Examples are Neodiprion lecontei nucleopolyhedrovirus (NeleNPV) against the red-headed pine sawfly (*Neodprion lecontei* (Fitch)), Orgyia pseudotsugata multicapsid nucleopolyhedrovirus (OpMNPV) against the Douglas-fir tussock moth (*Orgyia pseudotsugata* (McDunnough)), Lymantria dispar multicapsid nucleopolyhedrovirus (LdMNPV) against the gypsy moth (*Lymantria dispar* (L.)) and Neodiprion abietis nucleopolyhedrovirus (NeabNPV) against the balsam fir sawfly (*Neodiprion abietis* (Harris)) (Moreau and Lucarotti, 2007). The application area of baculovirus products to suppress forest pest

insects was relatively small compared with other control measures, such as *B. thuringiensis* (Bt) (Lacey et al., 2001).

Another example for the successful use of a baculovirus in biological plant protection is the application of Cydia pomonella granulovirus (CpGV) against the codling moth*, Cydia pomonella*, in apple, pear and walnut production (Lacey et al., 2008; Huber, 1998). Biological control agents based on CpGV are registered and commercially applied in more than 30 countries worldwide with more than 100,000 hectares of application area just in Europe (Gebhard et al., 2014; Eberle and Jehle, 2006).

The Anticarsia gemmatalis multiple nucleopolyhedrovirus (AgMNPV) has been used against the velvet bean caterpillar*, Anticarsia gemmatalis*, in soybean production in Brazil since the 1070s. The application area of AgMNPV has been as large as approx. 10% of the total soybean production area with up to two to three million hectares in the years 2003/2004 (Ferreira et al., 2014; Moscardi, 1999).

Many *Helicoverpa* (*Heliothis)* species, such as *Helicoverpa zea* and *Heliothis virescens*, have been controlled by NPVs in the USA between the years 1975 to 1980 with a total estimated treated area of over 1 million hectares but dropped substantially in 1981 (Ignoffo and Couch, 1981). *Helicoverpa armigera* was controlled on approximately 100,000 hectares in China in the late 1970s (Vlak and Hu, 1997; Zhang and Bai, 1992; Yi and Li, 1989) and in Russia (Filippov, 1990).

Phthorimaea operculella granulovirus (PhopGV) showed a high efficacy in protecting potato crops in the field as well as stored potato tubers (Alcázar et al., 1993; Raman et al., 1992). Therefore it was already used as biocontrol agent against PTM in potato fields and in storehouses in Yemen (Kroschel et al., 1996), Egypt, Tunisia (Smith and Bellotti, 1996) and several countries in Latin America *e.g.* Colombia, Peru, Costa Rica, Bolivia and Ecuador (Haase et al., 2015).

Baculoviruses: General Features

Baculoviruses are insect-specific enveloped viruses with circular, supercoiled double-stranded DNA genomes in the range of 80-180 kbp (Krell, 2008). The family of *Baculoviridae* is composed of four genera: *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus* (Herniou et al., 2011; Jehle et al., 2008).

Typically for baculoviruses, virions exist as two phenotypes (Herniou et al., 2011): occlusion-derived virions (ODV) are occluded in a crystalline protein matrix called occlusion body (OB). The OB morphology can be either polyhedral, ranging in size from 0.5 to 5 µm and containing many virions, as it is typical for the genera *Alphabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus,* or ovicylindrical (about 0.3 × 0.5 µm), containing only one virion (genus *Betabaculovirus*). The stick-shaped virions (*baculum*, Latin for stick) are eponymous for the family *Baculoviridae*. Virions consist of rod-shaped single or multiple nucleacapsids of 30 × 300 nm in size, where each nucleocapsid represents one copy of the virus genome (Krell, 2008). The second virion phenotype is generated when nucleocapsids bud through the plasma membrane at the surface of infected cells and are called budded virions (BV) (Figure 1). The major protein of the OB matrix is a virus-encoded polypeptide of 25–33 kDa. This protein is called polyhedrin for nucleopolyhedroviruses (*i.e.* alpha-, delta- and gammabaculoviruses) and granulin for granuloviruses (*i.e.* betabaculoviruses). The polyhedrin protein of deltabaculoviruses is serologically and genetically unrelated to OB proteins of the alpha-, beta- and

gammabaculoviruses (Herniou et al., 2011). A baculovirus genome encodes 100-200 proteins, whereas 38 gene homologs, the so-called baculovirus core genes, are shared by all alpha-, beta-, gamma- and deltabaculoviruses (Table 1) (Javed et al., 2017).

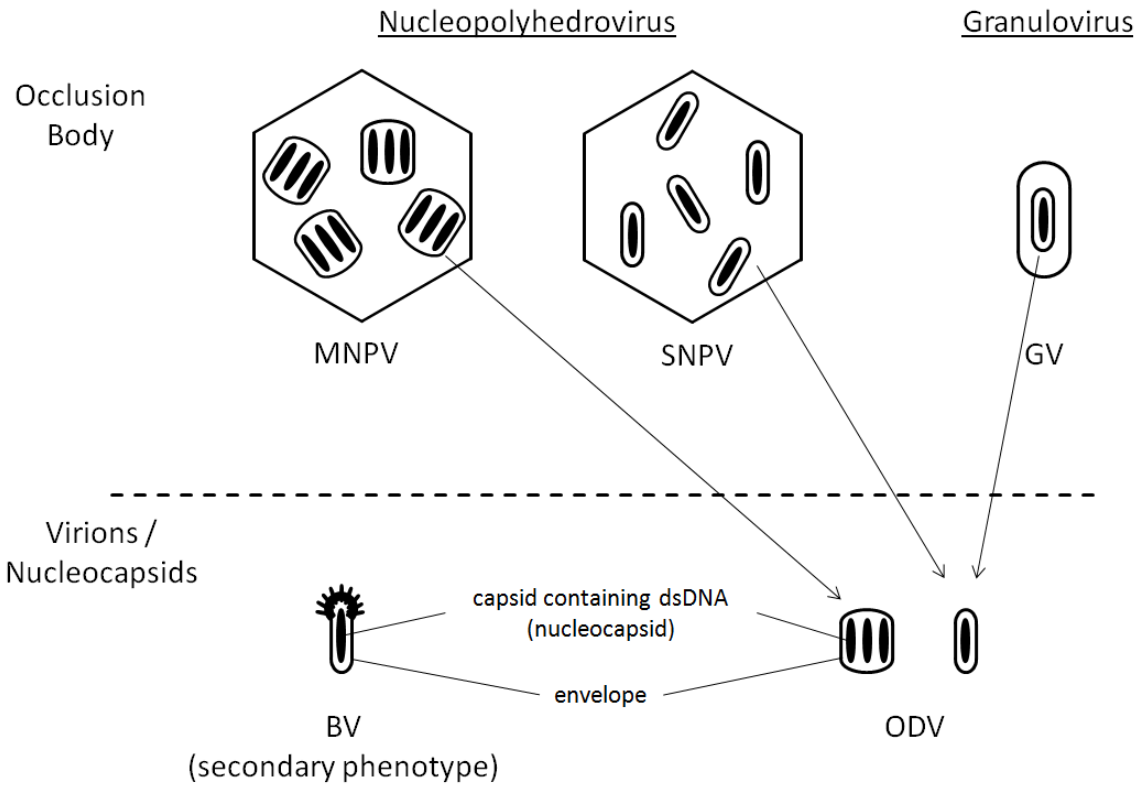


Figure 1. Baculovirus occlusion bodies, virions and nucleocapsids from the genera *Alphabaculovirus* (nucleopolyhedrovirus, NPV) and *Betabaculovirus* (granulovirus, GV) are illustrated. Virions of the NPV occlusion bodies may contain multiple nucleocapsids (MNPV) or single nucleocapsids (SNPV). Virions occur as two phenotypes: occlusion derived virions (ODV) or budded virions (BV). BV show a characteristic spike-like peplomere structure at one or two ends and contain one nucleocapsid, whereas ODVs can contain one nucleocapsid or more (changed after ICTV, 2014).

Table 1. List of 38 core genes shared with other baculoviruses (Javed et al., 2017).

01 - *helicase*	11 - *alkexo*	21 - *lef-8*	31 - *ac81*
02 - *pif4 ac96*	12 - *p74*	22 - *ac53*	32 - *vp91 p95*
03 - *38k*	13 - *p49 49k*	23 - *vp1054*	33 - *vp39*
04 - *lef-5*	14 - *odv-e18*	24 - *lef-9*	34 - *lef-4*
05 - *p6.9*	15 - *odv-e27*	25 - *DNApol*	35 - *p33*
06 - *p40*	16 - *pif-5 odv-e56*	26 - *desmoplakin*	36 - *p18*
07 - *p12 p48*	17 - *lef-2*	27 - *pif-6 ac68*	37 - *odv-e25*
08 - *odv-ec43*	18 - *lef-1*	28 - *vlf1*	38 - *pif-7*
09 - *pif-3*	19 - *pif-2*	29 - *ac78*	
10 - *pif-1*	20 - *p47*	30 - *gp41*	

Baculovirus Infection Cycle

Host larvae become infected *per os* by ingestion of virus OBs together with food or grooming (Figure 2a). Caused by the high alkaline pH (8-11) of the midgut, the OBs dissolve and thereby release ODVs (Volkman, 2008). After transition of the peritrophic membrane lining the midgut lumen, the ODVs attach to the epithelial cell surface followed by entry of the nucleocapsids into the midgut epithelial cells by membrane fusion. Once entered the cell, the nucleocapsids are transported to the nucleus where the transcription of early genes is initiated by the cellular RNA polymerase II (Friesen, 1997; O'Reilly et al., 1992). The transcription of delayed early genes requires the activation by early viral gene products expressed at the previous stage. So-called late expression factors (lefs) are needed for DNA replication and transcription of late and very late genes (Hefferon and Miller, 2002). In the late and very late phase of infection a virus-encoded RNA polymerase transcribes genes which encode structural proteins needed for virion production (Lu et al., 1997). The production of BVs leads to a secondary infection (Figure 2b) of additional cells and allows the virus to spread to other tissues, such as fat body (Lacey et al., 2011). The rate of BV production decreases in the very late phase and nucleocapsids were used to build ODVs which become occluded with major capsid protein Polyhedrin (alphabaculoviruses, gammabaculoviruses and deltabaculoviruses) or Granulin (betabaculoviruses) to form OBs in the infected host cell nucleus. To facilitate the release of the newly produced OBs to the environment, the virus encoded enzymes cathepsin and chitinase help to disintegrate the cuticula and to liquify larval cadavers in the final stage of infection. The release of OBs to the environment generates a source for infection of other host individuals (Hawtin et al., 1997).

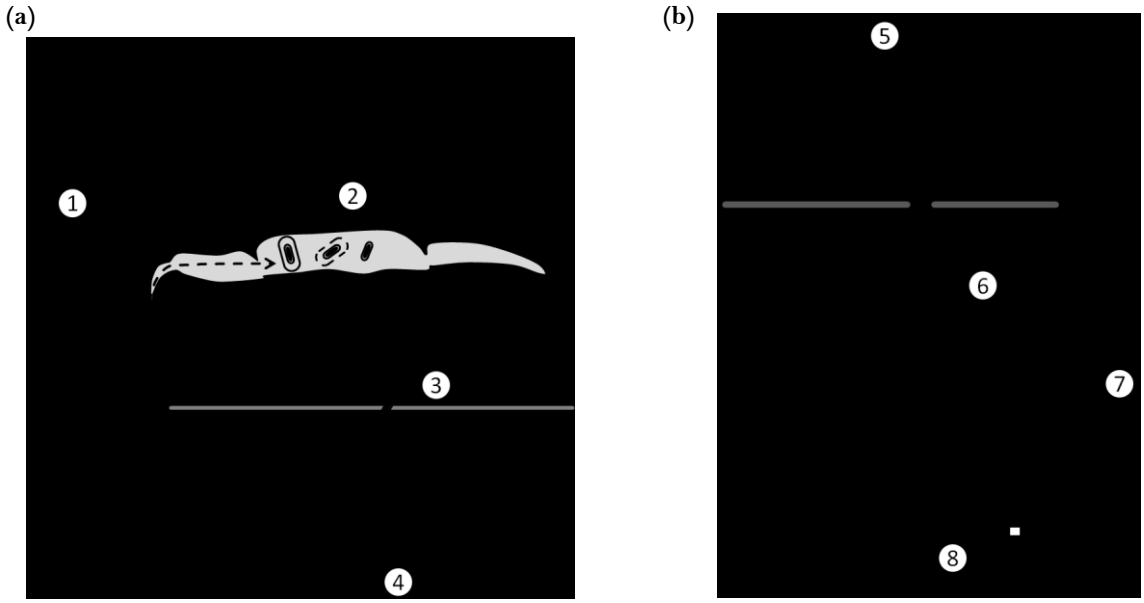


Figure 2. Baculovirus infection cycle with (a) a schematic primary infection by occlusion body (OB) and (b) secondary infection by budded virions (BV). Ingested OB start a new infection cycle (1). When OB are transported to the alkaline midgut of the host, proteinaceous matrix is dissolved and occlusion derived virions (ODV) are released (2). Virus and host encoded enzymes present in the OB degrade the peritrophic membrane allowing the ODV to attach to microvilli of midgut epithelial cells and to enter the cells by membrane fusion (3). The nucleocapsids (NC) are transported to and enter the nucleus, the locus of virus replication (4). New NC are assembled (5) and transported from the nucleus to the basal membrane from where they bud into the hemocoel (6) and become distributed via hemolymph or tracheae within the whole insect. The released BV can enter uninfected cells by endocytosis and start a secondary infection (7). Nucleocapsids enter the nucleus where new NC become assembled and embedded in OBs in the late stage of infection (8).

Baculovirus Covert Infection and Virus Transmission

Whereas overt infections result in heavily diseased larvae which succumb to baculovirus infection, covert infections can be survived and can become stably established in an insect population (Sait et al., 1994), characterized by the absence of visible signs of infection (Williams et al., 2017). Covert baculovirus infections can be found in many different insect populations, in the laboratory and in the field (Murillo et al., 2011; Erlandson, 2009; Burden et al., 2003; Fuxa et al., 1999; Kukan, 1999; Hughes et al., 1997). A covert baculovirus infection is a strategy for long-term stable infection of host populations and further virus transmission. Virus transmission can follow two different mechanisms: (1) an overt baculovirus outbreak can kill a large number of individuals from a population which will function as infective sources for other host insects either from the same or another generation (horizontal transmission); or (2) a covert baculovirus infection which does not result in the death of the infected individual and can therefore be transmitted from the infected parental generation to their offspring (vertical transmission) (Andrealis, 1987). Horizontal virus transmission through *per os* infection within one host generation and the following virus infection cycle are well characterized for many insect viruses, including polydnaviruses, rhabdoviruses (*e.g.* sigma viruses), picornaviruses

(*e.g.* dicistroviridae), nudiviruses and baculoviruses (Valles et al., 2017; Cory, 2015; Rohrmann, 2011; Burand, 1998). But the understanding of the mechanisms involved in vertical virus transmission, where virus transmission occurs from parents to offspring is still in its infancy (Kukan, 1999). In contrast to the acute infection in the horizontal virus transmission pathway, a vertical virus transmission often occurs with chronic infections within an insect population. It can appear either as persistent infection resulting in a continuous low level of virus replication after a primary infection, or as a latent infection where the primary infection, a low level reactivation and recurrent infection appears (Mocarski and Grakoui, 2008). The verification of covered infections is complicated because the time point of analysis is crucial and can be misleading. A persistent low level replication could also be a beginning acute infection at an early stage and a latent recurrent infection with zero virus replication between low level virus replication could also be a host without any virus infection at all.

Phthorimaea operculella granulovirus (PhopGV)

General Features

Phthorimaea operculella granulovirus (PhopGV) is a member of the genus *Betabaculovirus* and part of the family of *Baculoviridae*. One infective OB contains a single enveloped ODV (Funk et al., 1997) (Figure 3). The virion can appear in two different phenotypes: (a) the budded virus (BV) and (b) the occluded virus (ODV) (Figure 1).

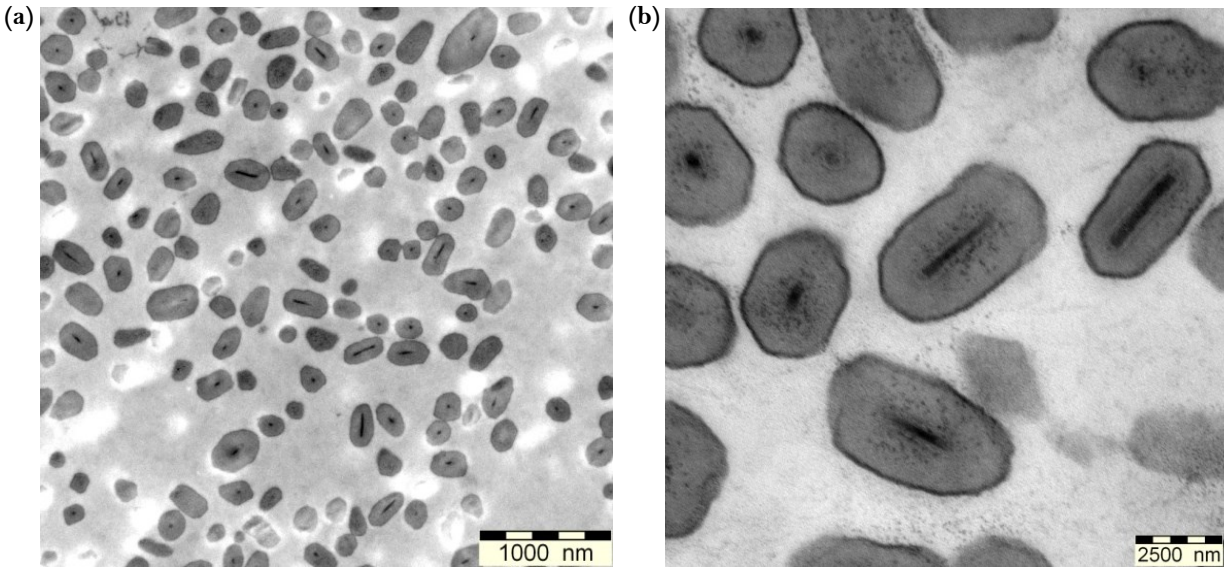


Figure 3. Transmission electron micrograph of Phthorimaea operculella granulovirus (PhopGV) at (a) low and (b) high magnification.

The genome of PhopGV consists of circular double-stranded DNA with a total length of about 119 kbp (Jukes et al., 2016; Croizier et al., 2002). PhopGV encodes 130 ORFs in total and shares 38 core genes with other baculovirus species (Javed et al., 2017) (Table 1). PhopGV can infect a number of different species of the Gelechiidae family, such as the three already introduced species *Phthorimaea operculella*, *Tecia solanivora*, *Tuta absoluta* (Lacey and Kroschel, 2009) and additionally species like *Symmetrischema tangolias* (Gyen), *Eurysacca quinoae* (Povolny) and *Paraschema detectendum* (Povolny) (Carpio et al., 2013; Povolny, 1967).

PhopGV is a “slow-killing” granulovirus (Gómez Valderrama et al., 2017; Federici, 1998), which means that infected larvae continue their development and die – independently from their stage of infection – in the last larval stage directly before pupation. This is possible because only some tissue parts like midgut, fat body and hypodermis become infected with virus (Lacey et al., 2011). In contrast, other granuloviruses like CpGV are considered as fast-killing viruses because they often kill their hosts *e.g.* the codling moth (*C. pomonella*), in the same larval stage of infection within 5-10 days (Hilton, 2008; Federici, 1997). Fast-killing baculoviruses infect comprehensive parts of the host tissue with fat body, epidermis, Malpighian tubules, tracheal matrix, hemocytes, and many other tissues to a lesser extent (Federici, 1997). Virus genes like ecdysteroid UDP–glucosyltransferase (*egt*) interfere with host moulting, resulting in a retarded development of infected larvae and allowing especially "slow-killing" viruses to gain a longer time to develop in the larval stage (O'Reilly, 1995). The host development becomes regulated by the inactivation of ecdysteroids through the activity of *egt* which catalyses the conjugation of sugars onto ecdyteroids (O'Reilly, 1995; O'Reilly and Miller, 1989). As a result, infected larvae are unable to pupate (Nakai et al., 2004; O'Reilly, 1995). This general effect was also reported for PhopGV infected larvae of the PTM, which completed development but failed to pupate, thus preventing development of future generations (Sporleder et al., 2005).

In case of PhopGV, ORF 129 (*egt*) can appear in five different types (I-V) distinguishable by the gene length (Jukes et al., 2016; Jukes et al., 2014; Carpio et al., 2013; Zeddam et al., 2013; Espinel-Correal et al., 2010) (Table 2).

Table 2. *Egt* types and lengths of ORF 129 of Phthorimaea operculella granulovirus.

Length (bp) of ORF 129	***egt* Type**
1305	I
1353	II
1086	III
1092	IV
861	V

Discovery and Historic Use of PhopGV

PhopGV was first isolated from infected potato tuber moth larvae in Sri Lanka and propagated in Australia (Reed, 1969); it has been found in various parts of the world in correlation with the distribution of its hosts (Espinel-Correal et al., 2010; Zeddam et al., 1999; Kroschel and Koch, 1996; Hunter et al., 1975).

Previously, PhopGV isolates of different geographical origins were characterized by restriction endonuclease (REN) analysis of genomic DNA preparations, with the result of only minor variability among different virus isolates (Carpio et al., 2013; Zeddam et al., 2013; Gómez-Bonilla et al., 2011; Zeddam et al., 1999; Vickers et al., 1991). On the other hand, PhopGV isolates differed in their biological activity against various hosts reported for PTM, *T. absoluta* and *T. solanivora* in laboratory bioassays (Gómez Valderrama et al., 2017; Zeddam et al., 2013; Espinel-Correal et al., 2010) indicating that there are differences in the genome which cannot be correlated with REN analysis patterns. Nevertheless, genotype mixtures could be identified by analysing submolar bands in DNA REN patterns of PhopGV isolates and were eventually confirmed by PCR and sequencing of particular regions of the viral genome (Zeddam et al., 2013; Espinel-Correal et al., 2010). These sequenced gene regions were namely *hypothetical protein* (PhopGV ORF 46), repeat region 9 (between *ODV-E25* (PhopGV ORF 83) and *hypothetical protein* (PhopGV ORF 84)), the area between *hypothetical proteins* PhopGV ORF 90 and ORF 91, as well as *egt* (PhopGV ORF 129).

The first whole genome sequence available at GenBank derived from isolate PhopGV-1346 (NC004062) from Tunisia (Croizier et al., 2002). Later a South African isolate PhopGV_SA (KU666536) has been sequenced (Jukes et al., 2016).

The practical use of PhopGV showed a high efficacy in protecting potato crops in the field as well as potato tubers under storage (Alcázar et al., 1993; Raman et al., 1992). The International Potato Center (CIP) in Peru was developing a microbial plant protection agent based on a PhopGV isolate. A number of 20 virus-infected larvae were grounded and mixed with 1 kg talc, used as a suspension in 1 l of water. A mortality of about 95% for PTM was reached applied at a dose of 5 kg per ton of stored potatoes (Raman et al., 1992, Raman et al., 1987). This strategy was then established in Bolivia, Ecuador and Colombia (Moscardi, 1999). A mixture of various selected PhopGV genotypes active against the target pests PTM and *T. solanivora* was established and registered in Colombia, recommended for the control of *T. solanivora* in stored potatoes (Haase et al., 2015; Espinel-Correal, 2012). Diseased PTM larvae collected in Costa Rica provided PhopGV-CR1, another isolate used for potato protection. Serial passage of PhopGV-CR1 over four generations in *T. solanivora* resulted in an increase in its virulence by about five-fold in three generations, suggesting a rapid adaptation to its alternate host and a decrease of damage of over 70% under storage conditions compared with the untreated controls (Gómez-Bonilla et al., 2011). In Brazil, an indigenous PhopGV isolate from PTM was characterized and evaluated against PTM and *T. absoluta* (Moura Mascarin et al., 2010). Combinations of OBs with commercial neem-oil based products (1×10^4 OBs/ml and 4 mg of azadirachtin/L) achieved high larval mortality of about 90%, whereas a talc-based virus formulation resulted in 100% larval mortality at 5×10^8 OBs/g and provided a better control efficiency on PTM than an aqueous virus suspension (Haase et al., 2015). Further, PhopGV was used as biocontrol agent in Yemen (Kroschel et al., 1996), Egypt and Tunisia (Smith and Bellotti, 1996) against PTM in potato fields and in storehouses.

Microsporidia in Plant Control

On the example of the gypsy moth *Lymantria dispar*, microsporidia are considered as important cofactor in maintaining population densities below tolerance threshold for a long period of time (Novotny, 1988; Weiser and Novotny, 1987). But only the microsporidium *Nosema locustae* has been registered as a biocontrol agent against grasshoppers in grasslands in the USA (Solter and Maddox, 1998).

Microsporidia

Microsporidia are obligate intracellular pathogens of the superphylum Opisthosporidia, a deep-branch clade of Holomycota related to the Fungi (Karpov et al., 2014; Keeling, 2014). More than 185 genera and over 1,300 species are described today that infect protists, invertebrates and vertebrates, including humans (Vavra and Lukes, 2013; Solter and Becnel, 2012). Microsporidia cause damages in apiaries, fisheries, and silk farms and cause severe disease in immune-compromised humans (Keeling and Fast, 2002).

The general life cycle of microsporidia can be divided into three phases: the infective phase, the proliferative phase, and the spore forming phase (Visvesvara, 2002). These intracellular parasites can only survive as infective spores outside of a host cell, protected by walls of protein and chitin (Kwak et al., 2013) (Figure 4a).

Spore size can range from 2-40 µm in diameter depending on the different species, however microsporidia infections cause generally chronic diseases and reduce the physiological and reproductive ability of their host (Corradi and Keeling, 2009). The coiled polar filament (Figure 4b) of microsporidia spores is used to inject the sporoplasm into the host cell upon spore germination. This polar filament and the feature of the diplokaryon arrangement of the nuclei clearly define microsporidia structurally (Vossbrinck and Debrunner-Vossbrinck, 2005). The number of polar filament coils is one criterion for discriminating *Nosema* species (Burges et al., 1974).

Microsporidia attack different groups of invertebrate and vertebrate hosts (Hernández-Velázquez et al., 2012). Many species of microsporidia infect arthropods, especially insects such as Lepidoptera and Coleoptera (Corradi and Keeling, 2009; Weiser, 2005; Solter and Maddox, 1998). Microsporidia cause effects that depend on the species and concentration; however, they generally produce weakness and eventually lead to death of infected host individuals. The chronic infection of a host is expressed by prolonged developmental time; reduced adult size, longevity, fecundity, mating and egg fertility; and increased mortality in all developmental stages (Novotny and Weiser, 1993). This infection can follow two routes that result in mortality, one resulted from the chronic effects produced by the exposure of larvae to low doses of spores which lead to microsporidiosis just before pupating and the other route is the intake of a large number of spores and a resulting damage of the gut caused by introduction of a large number of spore polar filaments (Fuxa, 1981). Transmission can be orally via spores or vertically via eggs. Orally ingested spores germinate in the midgut of insects and infect the epithelial cells by a hollow polar filament. Infected cells multiply the microsporidia and form new spores, which are then transmitted to new hosts (Eilenberg et al., 2015). Infection does not always lead to mortality, a vertical transovarially transmission can lead to long-term effects of reduction in fecundity and susceptibility to other stress situations (Inglis et al., 2000).

Nosema

The genus *Nosema* (Microsporidia: Nosematidae) is associated with twelve different orders of insects and comprises more than 150 described species (Becnel and Andrealis, 2014). The first described microsporidium of the genus *Nosema* was *Nosema bombycis* (Nägeli), the causal agent of the pébrine disease in the silkworm, *Bombyx mori* L. (Becnel and Andrealis, 2014; Vavra and Lukes, 2013). The transmission of *Nosema* can be horizontally through consumption of spores (Campbell et al., 2007) and vertically from the female parent to offspring (van Frankenhuyzen et al, 2007; Bauer and Nordin, 1989; Thomson, 1957). *Nosema* spores have a diplokaryon nucleus, endospore and exospore wall and developed organelles (Terry et al., 1999).

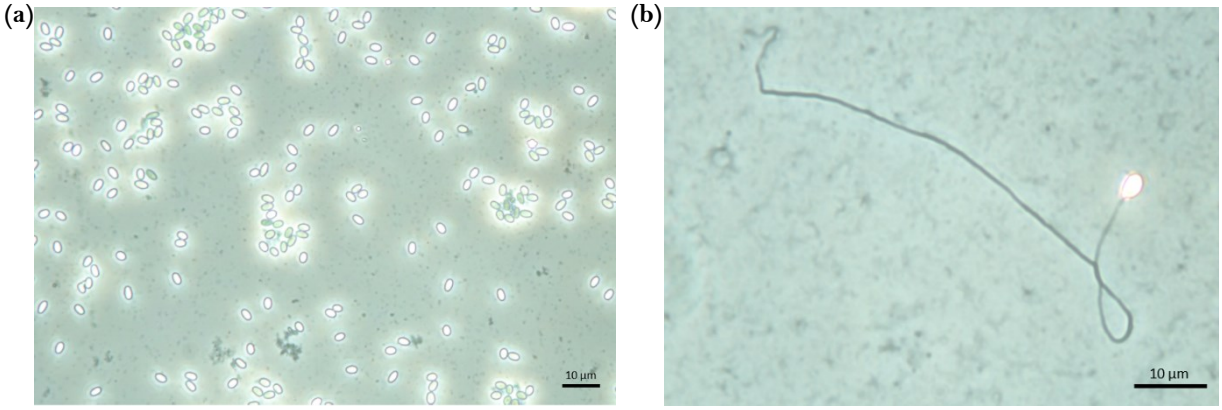


Figure 4. (a) Light microscopy pictures of *Nosema* sp Phop spores and (b) a single spore with an expelled polar filament of 109 µm length.

For differentiation of the "true *Nosema*" group from the other microsporidia (Solter and Becnel, 2012; Huang et al., 2004) rDNA loci have been used with the internal transcribed spacer (ITS) and the small subunit (SSU) rRNA gene. Additionally, the RNA polymerase II subunit (RPB1) was used to prove the relationship between microsporidia and fungi (Hirt et al., 1999) or for identification of microsporidian genera or species (Luo et al., 2014; Gisder and Genersch, 2013; Vavra et al., 2006; Cheney et al., 2001). Members of the "true *Nosema*" clade are characterized by having a reversed arrangement of the ribosomal subunits with a LSU-ITS-SSU configuration instead of the SSU-ITS-LSU arrangement found in other microsporidia species. This reversed order of ribosomal subunits was first identified in *N. bombycis* (Huang et al., 2004), the type species representing the "true *Nosema*" clade.

Aim of the Thesis

This thesis is addressed to investigate the diversity of Phthorimaea operculella granulovirus by the characterization of isolates derived from virus collections from four different continents (Africa, South America, Asia and Europe). The characterization methods should be molecular, phylogenetic, whole genome analysis and determination of the biological activity of the selected PhopGV isolates against the host *P. operculella*. In addition, the interaction of PhopGV co-infecting the same larva and virus infection under the presence of a *Nosema* sp. present in the *P. operculella* larvae should be investigated. Furthermore, the virus-host interaction should be complemented by the comparison of the response of two different *P. operculella* populations to the same PhopGV isolate.

Chapter I comprises a general introduction to the subject of this thesis.

In Chapter II the biological activity of nine PhopGV isolates against *P. operculella* was compared by determining the median lethal concentration (LC_{50}) and median lethal time (LT_{50}). The discriminating concentration (LC_{95}) was determined for *P. operculella* strain Phop-IT, to infect a second *P. operculella* population (Phop-TN). The question should be anwered to which extend virulence of PhopGV is isolate but also host dependent.

In Chapter III the genetic diversity of PhopGV is elucidated. Twelve complete genome sequences of PhopGV were determined by Illumina Next Generation Sequencing (NGS). The question should be answerd how the different PhopGV isolates are related to each other. A thorough analysis of the sequencing data allowed developing a new grouping system based on the information gained by whole genome sequencing.

Chapter IV is about the effects of a covert infection with an internal PhopGV in insect populations of *P. operculella*. An internal virus isolate has been purified from the *P. operculella* laboratory colony Phop-IT and should be characterized on the genetic level. The interaction of this most of the time covert virus with other PhopGV isolates should be tested. Parameters, such as crowding of larvae, should be tested for their impact on activating the covert virus. It was further addressed if co-infection together with other PhopGV isolates was possible or if there is a superinfection exclusion.

Chapter V describes the potential interaction of PhopGV with a microsporidium (*Nosema* sp. Phop) in larvae of *P. operculella*. Mortality after co-infections of the most virulent (against Phop-IT) PhopGV-GR1.1 and *Nosema* sp. Phop are compared to the mortality values determined for PhopGV-GR1.1 alone. The chapter aimed to provide an initial identification and characterization of *Nosema* sp. Phop by molecular and electron microscopic tools.

Chapter VI comprises a general discussion of the results of the chapters II to V.

Chapter II

Biological Activity of PhopGV Isolates in Terms of Median Lethal Concentration (LC_{50}) and Median Lethal Time (LT_{50}) in the Host System *Phthorimaea operculella*

Abstract

Nine isolates of Phthorimaea operculella granulovirus (PhopGV) were tested for their biological activity in terms of median lethal concentration (LC_{50}) against a laboratory strain of *P. operculella* originating from Italy (Phop-IT). Whereas the majority of the tested isolates did not show a high virulence against Phop-IT after 14 dpi (slow-killing virus), one isolate PhopGV-GR1.1 showed a clear effect with $LT_{50} = 2.17 \times 10^3$ OB/ml. This isolate fulfilled the requirements for the determination of the median lethal time (LT_{50}) with a LT_{50} value of 10 days. It was demonstrated that pupation of test animals was inhibited by infection with a PhopGV. The estimated discriminating concentration (LC_{95}) for Phop-IT was tested against a second laboratory strain of *P. operculella* originating from Tunisia (Phop-TN). The comparison of two different *P. operculella* strains showed, that virulence of different PhopGV isolates seems to be not only isolate but also host dependet.

Introduction

The potato tuber moth (PTM) *Phthorimaea operculella* (Zeller) is considered the most damaging potato pest in tropical and subtropical agro-ecosystems (Lacey and Kroschel, 2009). Its distribution is reported in more than 90 countries worldwide and the appearance of PTM is spreading to Mediterranean and temperate regions (Kroschel and Sporleder, 2006; Sporleder at al., 2004). In the field, PTM females lay their eggs on the plant, usually on leaves, throughout the growing season. Hatching larvae mine leaves, stems and petioles causing irregular galleries (Gómez-Bonilla et al., 2011). Larvae also attack potato tubers by excavating tunnels mainly under storage conditions (Rondon, 2010). The mining larvae cause severe damage to tubers in the field and in storage, where losses may account for up to 100% under non-refrigerated conditions (Rondon, 2010; von Arx et al., 1987; Raman et al, 1987). Besides potato (*Solanum tuberosum* L.), a number of other solanaceous plants are damaged by *P. operculella, e.g.* tomato (*Lycopersicon esculentum* L.), capsicum (*Capsicum anuum* L.), eggplant (*Solanum melogena* L.) and tobacco (*Nicotiana tabacum* L.) (Lacey and Kroschel, 2009).

Attempts to control PTM with chemical pesticides have caused the development of multiple resistances to several organophosphorus and synthetic pyrethroids (Dogramaci and Tingey, 2008; Sour, 2008; Shelton et al., 1981). Alternatives for PTM control are strongly needed and baculoviruses appear as promising candidates as active ingredient for biocontrol agents, as evidenced by successful experiences previously reported (Haase et al., 2015). Phthorimaea operculella granulovirus (PhopGV) is a slow-killing baculovirus (Gómes Valderrema et al., 2017) but the higher transmission efficiency can provide a better long-term effect on lowering pest insect populations

compared to fast-killing baculoviruses (Takahashi et al., 2015). PTM larvae infected with PhopGV typically complete larval development but fail to pupate, thus preventing development of future generations (Sporleder et al., 2005).

In this study nine isolates of PhopGV have been tested for their biological activity against an Italian strain of *P. operculella* (Phop-IT). The median lethal concentration (LC_{50}) and the median lethal time (LT_{50}) were determined. The discriminating dose (LC_{95}) of two PhopGV isolates which showed high and low virulence against Phop-IT were tested against a second PTM strain from Tunisia (Phop-TN), to test the influence of a different host strain on virus caused mortality.

Material and Methods

Insect Rearing

A rearing of the potato tuber moth *P. operculella* was established at the Institute for Biological Control, JKI Darmstadt, in 2014. The insects originated from a laboratory colony isolated in Emilia Romagna, Ravenna, Italy (COOP. TERREMERSE, Bagnacavallo) and was termed Phop-IT. The larvae were kept on potato slices at 26 °C and under 16/8 h light/dark photoperiod until pupation. Potato slices were placed on sand to allow pupation outside of the potato and to facilitate the collection of the pupae with a mesh. After hatching, the adults were transferred to open plastic cylinders (⌀ 24.5 cm, height = 18 cm). The cylinders were lined with a dark plastic bag in order to protect the insects from solar irradiance. The top end of each cylinder was covered with a fine gauze and an additional layer of filter paper. The gauze allowed egg laying on the filter paper. This technique allowed an exchange of the egg paper with a fresh one without opening of the cylinder. The adults were fed with 10% sucrose solution. After the collection of the egg paper, the eggs were incubated at either 20 °C or 26 °C, to regulate the hatching day of the neonate larvae. Neonates were used for successive rearing cycles as well as bioassay experiments.

In addition to the strain Phop-IT, an additional *P. operculella* laboratory rearing was established from insects collected from a potato storage room in Tunisia (Phop-TN). This insect population was kindly provided by the Culture Defense Directorate of the Center Tunisia ("Direction de défense de culture du centre"). The insect rearing was kept separately but under the same rearing, temperature and light conditions. To avoid an interaction between the different insect strains, the two rearings were maintained not only spatially separated from each other but they were also maintained by different persons.

PhopGV Isolates

Different PhopGV isolates (Table 1) were obtained either from infected larvae or as purified occlusion body (OB) suspension. All obtained virus samples were initially propagated in the established laboratory colony of *P. operculella* to obtain a sufficient OB stock of all isolates. Isolates that were propagated in one cycle in *P. operculella* were given the suffix ".1" to their isolates names. If a second round of propagation followed, the suffix ".2" was added.

Isolate PhopGV-YM.1 was collected in Yemen in 1989 which was followed by several publications describing the biological activity and a possible role in the integrated pest management for controlling PTM in Yemen (Kroschel and Koch, 1996; Kroschel, 1995). Isolate PhopGV-#1390.2 was obtained from Public University of Navarra (UPNA) in Pamplona, Spain and was originally isolated from Peruvian *P. operculella* larvae (Vickers et al., 1991). The isolates PhopGV-GR1.1 and PhopGV-GR2.1 were first passages of isolates derived from soil samples collected from a potato production area in Greece in the framework of the BIOCOMES project in 2014. Isolates PhopGV-IT1.1 and PhopGV-IT2.1 derived from soil samples from tomato production in Italy, collected during the same project in 2014. The laboratory strain PhopGV-LS1.1 was obtained from Horticulture Research International (HRI) in Warwick/Wellesbourne. Two of the passaged isolates namely PhopGV-CR3.1 and PhopGV-CR5.1 had their origins in Costa Rica. These two isolates were previously described as PhopGV-CR3 and PhopGV-CR5 and characterized by *egt* sequence analysis and whole DNA REN digests (Zeddam et al., 2013; Gómez-Bonilla et al., 2011).

Virus Propagation

The surface of each potato disc (4.3 cm Ø, 0.5 cm thickness) was inoculated with 200 µl PhopGV OB suspension (1×10^4 OB/ml). Twenty neonate larvae of *P. operculella* were transferred onto one potato disc using a fine paint brush. The potato discs were kept at 26 °C, 60% RH and 16/8 h light/dark photoperiod. After six days the inoculated larvae showed typical baculovirus infection symptoms like loss of mobility, decreased feeding rate and change in colour from green to bright white followed by sluggishness and flaccidity (Gómes Valderrama et al., 2017; Lacey et al., 2011; Briese, 1981; Reed, 1969). The potato discs were examined daily and dead larvae were collected.

Purification of Occlusion Bodies from Infected Larvae

Virus OB were isolated from infected larvae according to the protocol of Smith and Crook (1988) with some changes for upscaling from single larvae to pooled larvae samples. To obtain an OB stock for all following experiments, approximately 50-60 virus infected insect cadavers were homogenized in 15 ml dH_2O using an Ultra-Turrax (IKA T25, Janke & Kunkel Labortechnik, Staufen, Germany). SDS was added to a final concentration of 0.5%, followed by an incubation for 30 min on ice. Subsequently the suspension was incubated for 3 min in an ultrasonic water bath. Larger larval debris was removed from the suspension by filtration through a double layer of cotton. The filter was washed with 4 ml additional volumes of 0.5% SDS (or 50 mM Tris pH 8.0) and dH_2O, to rinse remaining OBs from the filter into the collection tube. Then the collected suspension was centrifuged for 15 min at 22,000 × g at 12 °C. The supernatant was removed and the pellet was washed two times with dH_2O before resuspending the obtained pellet in 2 ml dH_2O. A discontinuous glycerol gradient 80/70/60/55/50% (v/v) was used to purify the OB. After centrifugation for 45 min at 3,200 × g (swinging bucket rotor) the remaining supernatant and the first three layers of the gradient (50-60%) were collected and washed with water (15 min at 22,000 × g). OBs were resuspended in 2 ml dH_2O and centrifuged for 15 min at 20,800 × g. The final OB pellet was then resuspended in 500 µl dH_2O and stored at -20 °C.

OB Quantification

The OB concentration was determined using a Petroff Hausser counting chamber (depth 0.02 mm) (Hausser Scientific, Horsham, Pennsylvania, USA) in the dark field optic of a light microscope (Leica, DMRBE, Leica Microsystems GmbH, Wetzlar, Germany). The concentration was calculated based on the mean of three independent counting steps of an appropriate dilution of each PhopGV isolate. This procedure of virus titration was repeated before the beginning of every experiment with a given isolate.

Biological Activity of PhopGV Isolates

In order to determine the biological activity (LC_{50}) of the different PhopGV isolates, neonates of *P. operculella* were subjected to full range bioassays using PhopGV suspensions ranging from 10^2-10^8 OB/ml to inoculate the surface of a potato disc (4.3 cm Ø and 0.5 cm thickness). The potato discs were parallel cut several times with a knife in order to facilitate the finding of the test larvae at the end of the bioassay. The surface of each potato disc was covered with 200 µl of the respected virus suspension or with H_2O for the untreated control group. Twenty neonate larvae were placed on each potato slice using a fine brush (Figure 1). Each of the potato slices was kept in a Petri dish and incubated at 28 °C and 16/8 h light/dark photoperiod. Mortality data were determined after 14 days by collecting the surviving larvae and pupae.

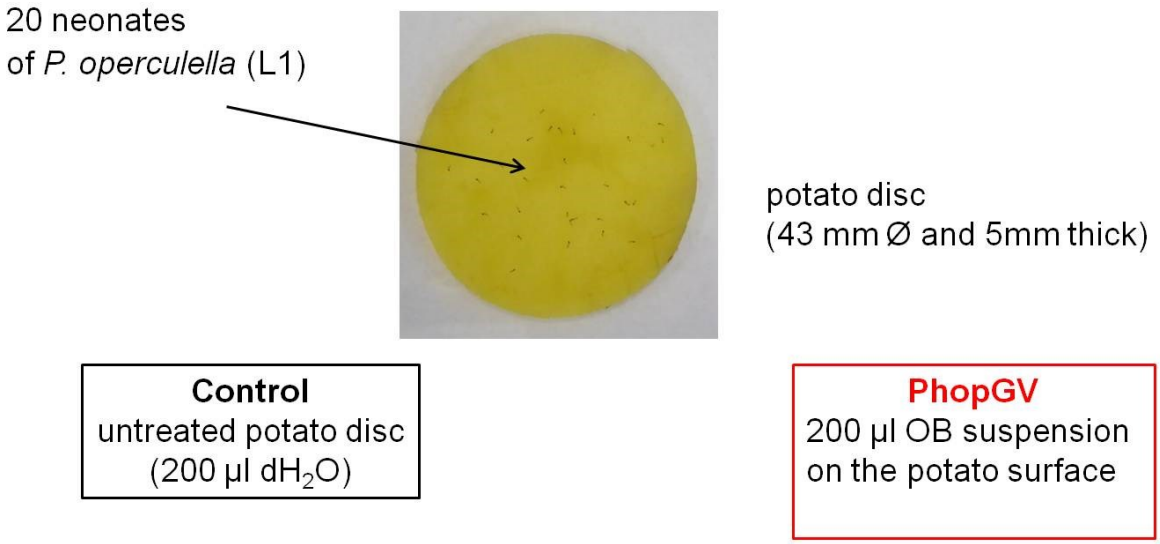


Figure 1. Picture of a potato disc as used for bioassays with different PhopGV isolates against neonates of *P. operculella*.

In addition, the median lethal time (LT_{50}) was determined for several PhopGV isolates using a single virus concentration of a virus isolate causing 80% mortality after 14 days, in neonates of the *P. operculella* laboratory strain Phop-IT. For each day of evaluation (day: 6, 7, 8, 9, 10, 11, 12, 13, 14) three petri dishes with each 20 neonates, were prepared and mortality was estimated. After evaluation, the petri dishes were discharged.

Statistical Analyses, ToxRat Probit

ToxRat Standard software (ToxRat Standard Version 3.2.1, ToxRat Solutions GmbH, Alsdorf, Germany) was used for statisitcal analysis, the calculation of the median lethal concentration (LC_{50}) and slopes of the regression lines. Further, this program was used to determine the median lethal time (LT_{50}).

Results

Estimation of Median Lethal Concentration (LC_{50})

A number of nine PhopGV isolates were tested in full range bioassays using virus suspensions ranging from 10^3-10^8 OB/ml to infect *P. operculella* neonates on surface contaminated potato slices. Mortality data were collected after 14 days corrected for control mortality according to Abbott (1925) and subjected to Probit analysis (Table 1).

Table 1. Median lethal concentration (LC_{50}) and Probit analysis parameters for different PhopGV isolates tested against neonates of *P. operculella* in 14-days bioassays at 28 °C and 16/8 h light/dark photoperiod. Mortality data of treatments were corrected according to Abbott (1925). Three independent replicates were performed for each isolate.

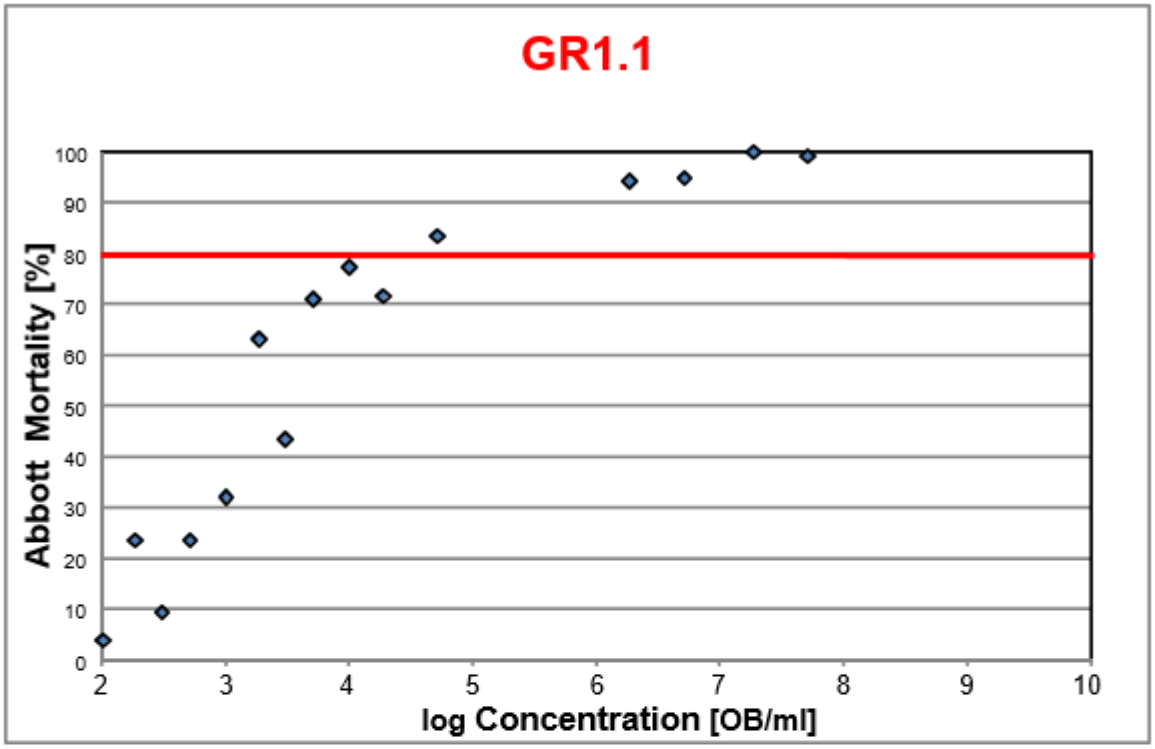
PhopGV Isolate	Origin	n Number of Tested Insects	(%) Control Mortality	LC_{50} [OB/ml] (95% Fiducial Limits)	Slope	Chi^2 (df)	Probit Linear Function
Ym.1	**Yemen**	1480	44.9	1.19×10^{10} (n.d.)	0.11*	145.74 (9)	-
#1390.2.1	**Peru**	1072	45.0	1.55×10^{4} (0.00 - 47.92)	0.25	50.71 (5)	y = 0.25 x + 4
GR1.1	**Greece**	2575	35.5	2.17×10^{3} (1.29 - 3.37)	0.77	40.90 (13)	y = 0.77 x + 2.4
GR2.1		1600	48.8	1.88×10^{6} (0.06 - n.d.)	0.33	81.25 (5)	y = 0.33 x + 2.9
IT1.1	**Italy**	1025	30.6	2.94×10^{6} (1.33 - 8.20)	0.36	8.32 (4)	y = 0.36 x + 2.7
IT2.1		1195	40.2	8.58×10^{4} (0.18 - 44.32)	0.50	219.07 (8)	y = 0.50 x + 2.5
LS1.1	**Laboratory**	1515	26.0	1.73×10^{13} (n.d.)	0.09*	31.24 (6)	-
CR 3.1	**Costa Rica**	980	39.2	8.67×10^{8} (n.d.)	0.20*	46.22 (5)	-
CR 5.1		1320	31.2	7.5×10^{16} (n.d.)	0.06*	156.6 (14)	-

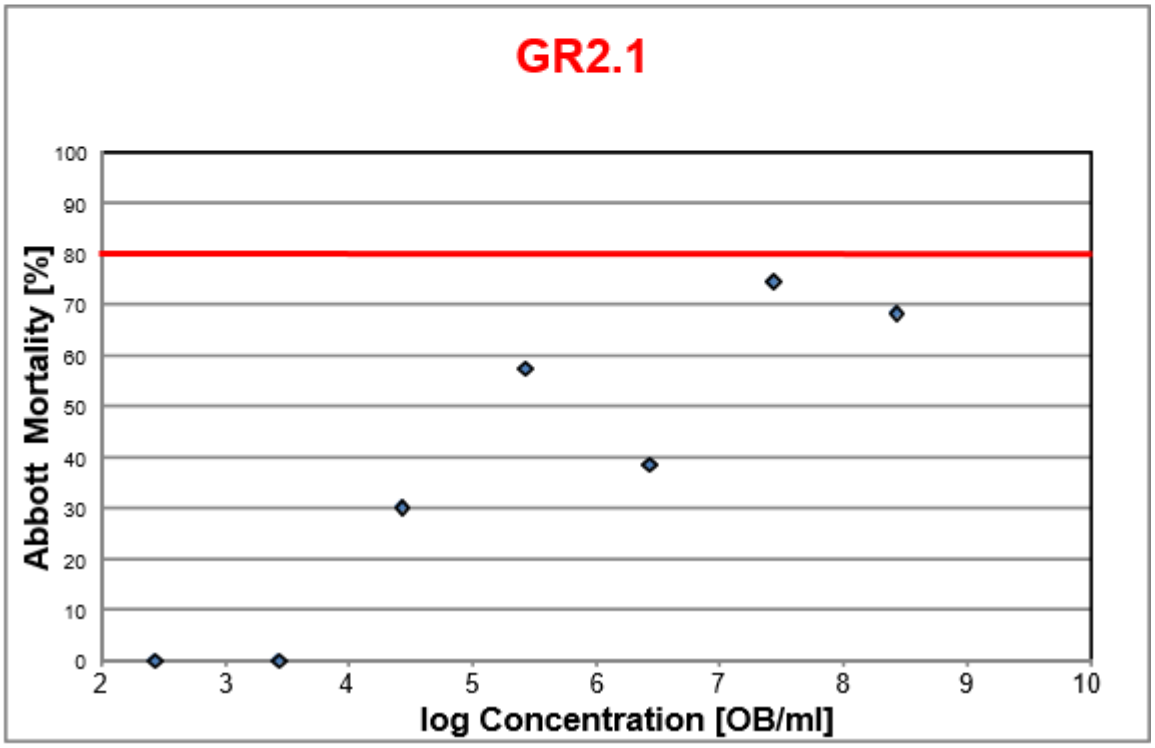
* No meaningful concentration/response was found (p(F) > 0.05; *i.e.* slope of the relationship is not significant different from zero.

Isolate PhopGV-GR1.1 showed the highest activity with a LC_{50} value of 2.17×10^3 OB/ml whereas the LC_{50} values of the isolates PhopGV-#1390.2.1, PhopGV-LS2.1, PhopGV-IT1.1, PhopGV-IT2.1 and PhopGV-GR2.1 were 7 to 1000 times higher. PhopGV-#1390.2.1 showed a relatively low LC_{50} concentration (1.55×10^4 OB/ml) compared to most of the other isolates, but a shallow slope (0.25).

Isolate LS1.1 was causing mortalities not higher than 50%, even at the highest concentration (10^{10} OB/ml). Significant estimation of slope (not significant different from zero) and LC_{50} value were not possible for PhopGV-LS1.1, PhopGV-YM.1, PhopGV-CR3.1 and PhopGV-CR 5.1., because of their low biological activity versus the *P. operculella* laboratory strain Phop-IT.

The concentration-response relationship of the three most active isolates, PhopGV-GR1.1, PhopGV-IT2.1 and PhopGV-GR2.1, and a weak PhopGV-LS1.1 is demonstrated in Figure 2 and served to select suitable isolates for the LT_{50} trials causing a final mortality of at least 80%. The red lines in the diagrams of Figure 2 indicate the 80% mortality level and reveal clearly that only GR1.1 and IT2.1 are reasonable isolates to be used for a determination of the lethal time (LT_{50}).





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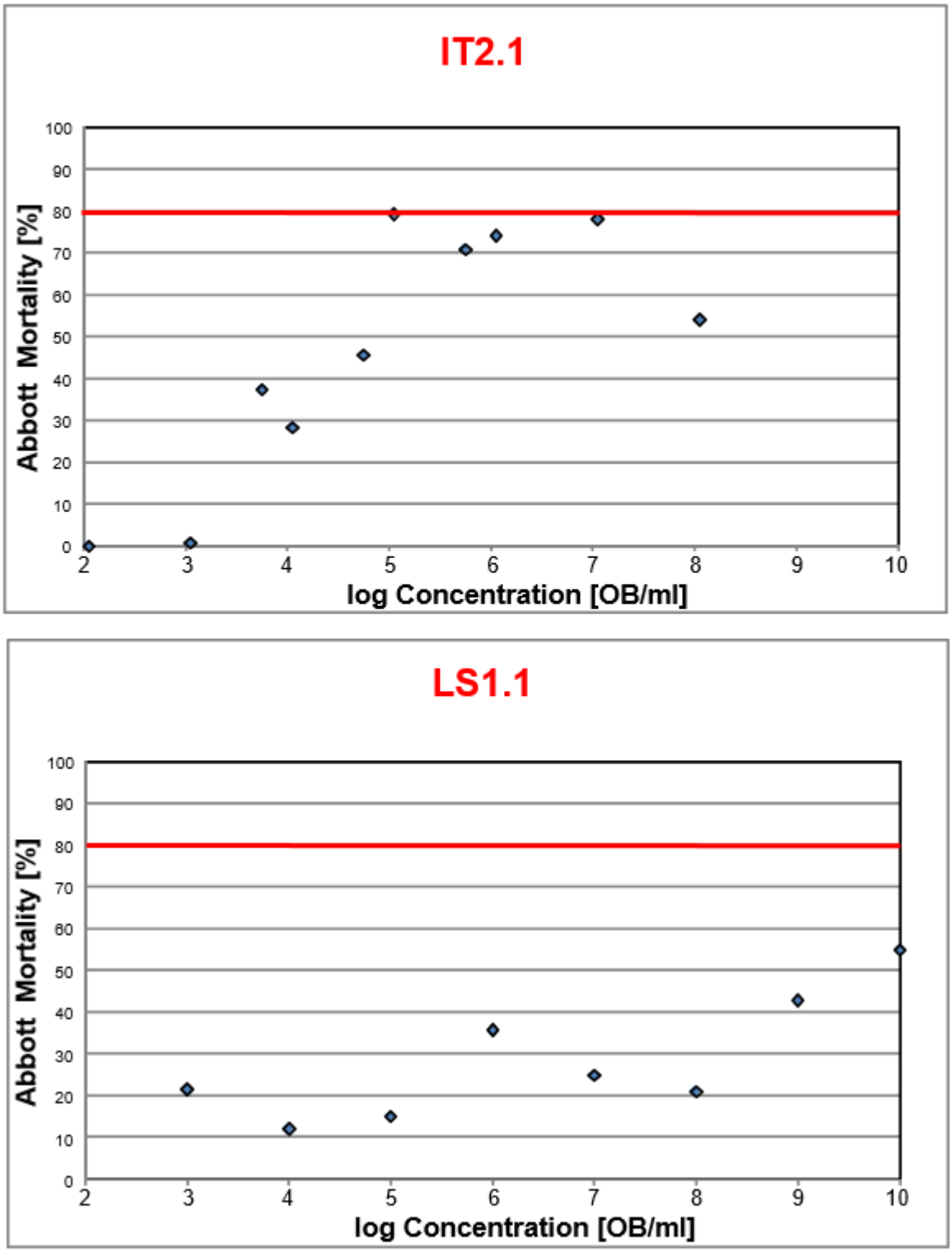

Figure 2. Results from 14-day full range bioassays with neonate *P. operculella* larvae on potato discs. Shown are the concentration-response relationships with Abbott (1925) corrected mortality data. The 80% mortality level is indicated by a red line for four isolates PhopGV-GR1.1, PhopGV-GR2.1, PhopGV-LS2.1 and PhopGV-LS1.1.

Survival Rate of *P. operculella* Infected With PhopGV-GR1.1

In order to determine the conditions for the LT_{50} bioassays a preliminary test with isolate PhopGV-GR1.1 was performed (Figure 3) with focus on the incubation time when virus-killed larvae occurred first (Figure 4). These results were used to design the following experiments and should help to choose the standardized evaluation time points of the LT_{50} bioassays.

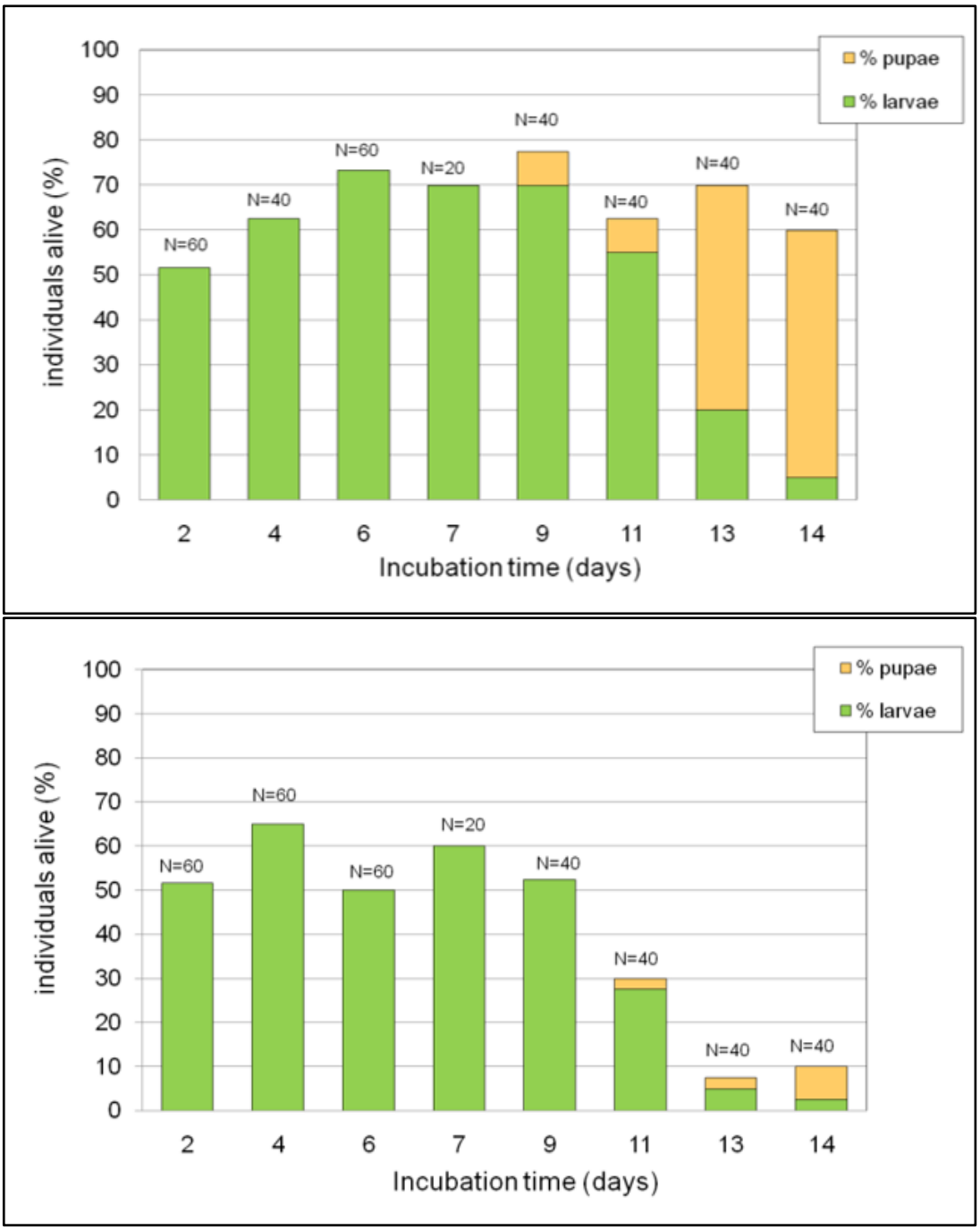

Figure 3. Survival rate (%) between day 2 to 14 of neonate *P. operculella* larvae after applying a LC_{80} concentration (1.3×10^4 OB/ml) of isolate PhopGV-GR1.1 (lower panel) and the control group (upper panel) on potato discs at 28 °C.

In the bioassays with potato discs, the mortality of the control group differed in general between 30% and 50%. The low rate of individuals found alive at day 2 seemed to be attributed to the difficulty to detect the very small larvae. The first decrease of the survival rate was visible between day 9 and 11 (Figure 3). In the following two plots additional information, including the rate of dead and virus-killed larvae are presented (Figure 4).

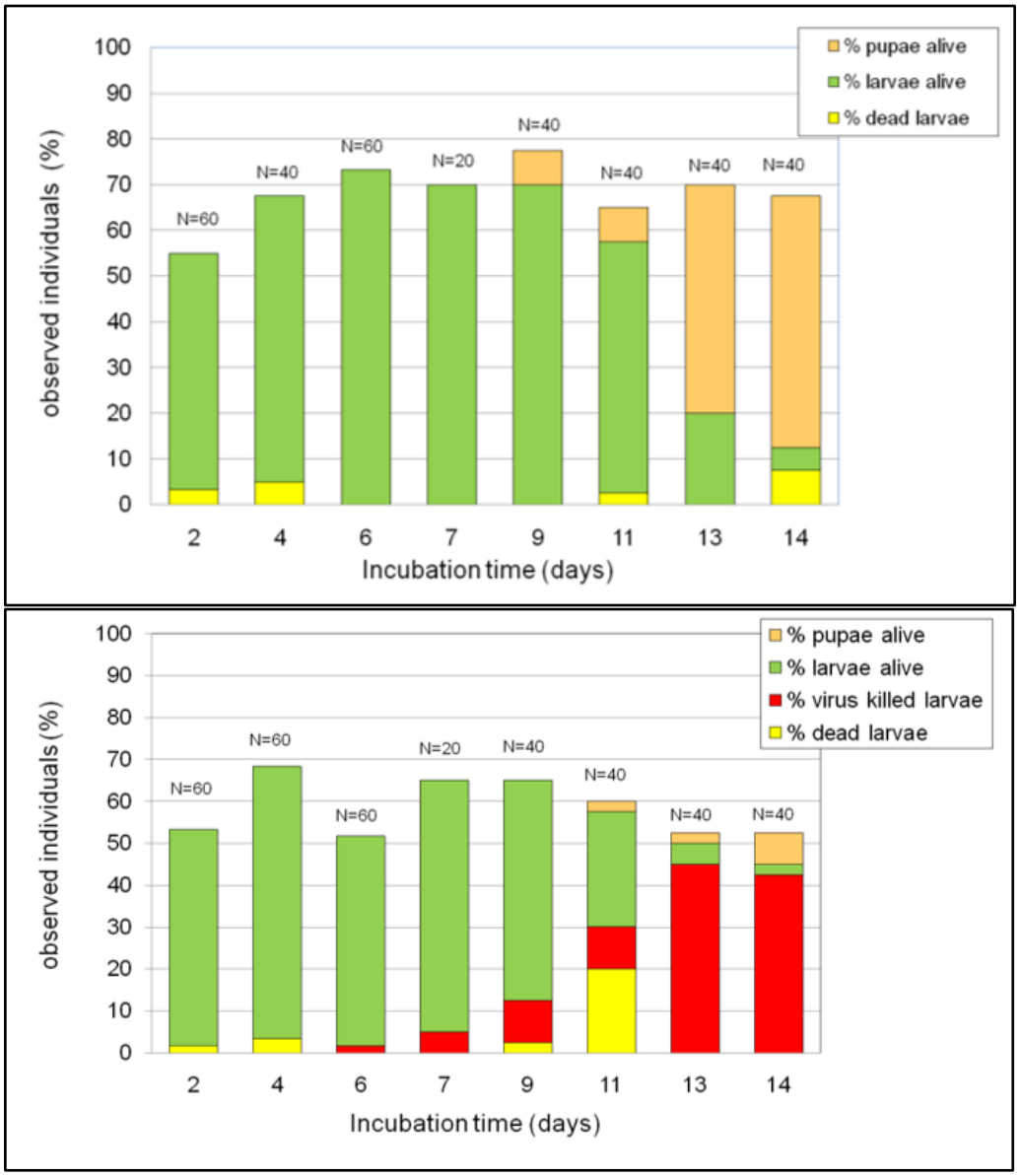

Figure 4. Survival rate (%) of *P. operculella* larvae treated with PhopGV-GR1.1 (1.3×10^4 OB/ml) (lower panel) and in the control group (upper panel) between day 2 and 14. The percent of all observed individuals including living larvae and pupae, dead larvae and virus killed larvae are given in the diagrams.

PhopGV killed larvae were first observed at day 6 (Figure 4) with a continuously increasing number of killed larvae until day 13. For the application of the LT_{50} assays, mortality was scored daily between day 6 and 14.

Estimation of Median Lethal Time (LT_{50})

Only two isolates, PhopGV-GR1.1 and -IT2.1, fulfilled the requirements for the determination of the LT_{50} (see Figure 2). Both showed a significant slope and a low LC_{50} concentration in full range bioassays. But only isolate PhopGV-GR1.1 showed a time/mortality response by application of 1.3×10^4 OB/ml (LC_{80}) against neonate *P. operculella* larvae (Figure 5).

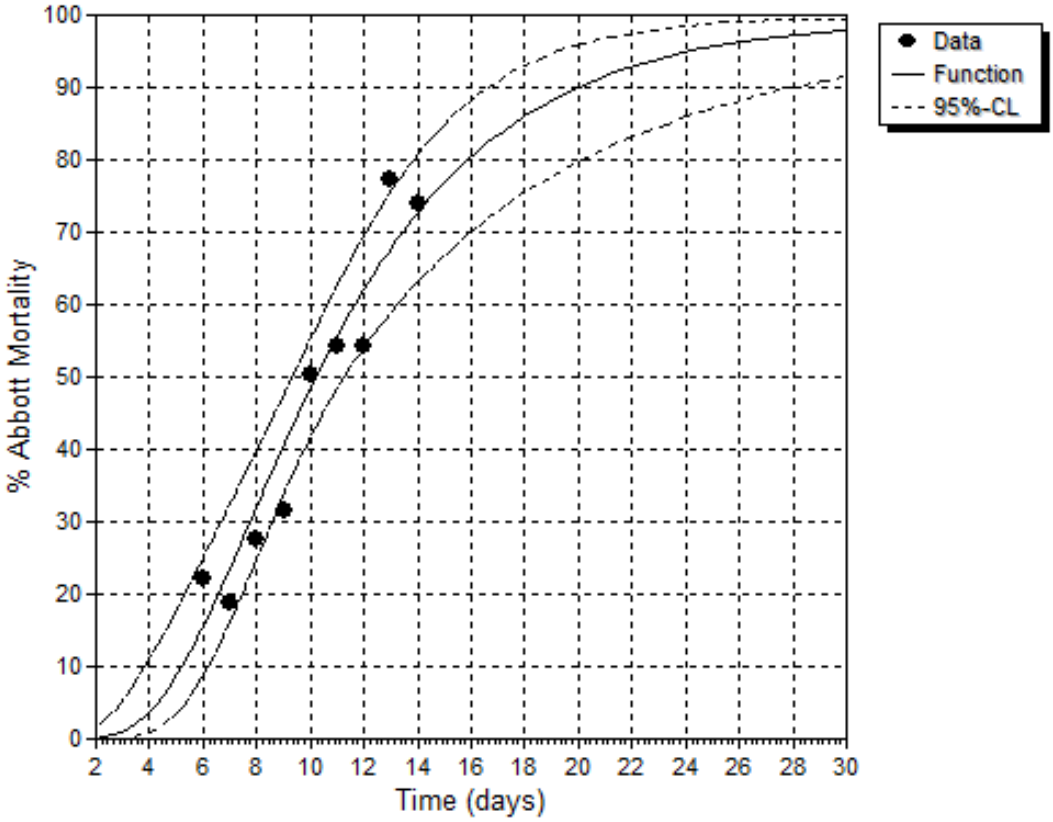

Figure 5. Time/mortality curve of isolate GR1.1 against neonate *P. operculella* larvae (n = 1,360). The diagram combines the data of three independent replicates of bioassays with a LC_{80} concentration (1.3×10^4 OB/ml). Each bioassay was evaluated from day 6 to 14. The larvae were incubated at 28 °C and 16/8 h light/dark photoperiod, during the experiment. The mortality was corrected according to Abbott (1925) before plotting.

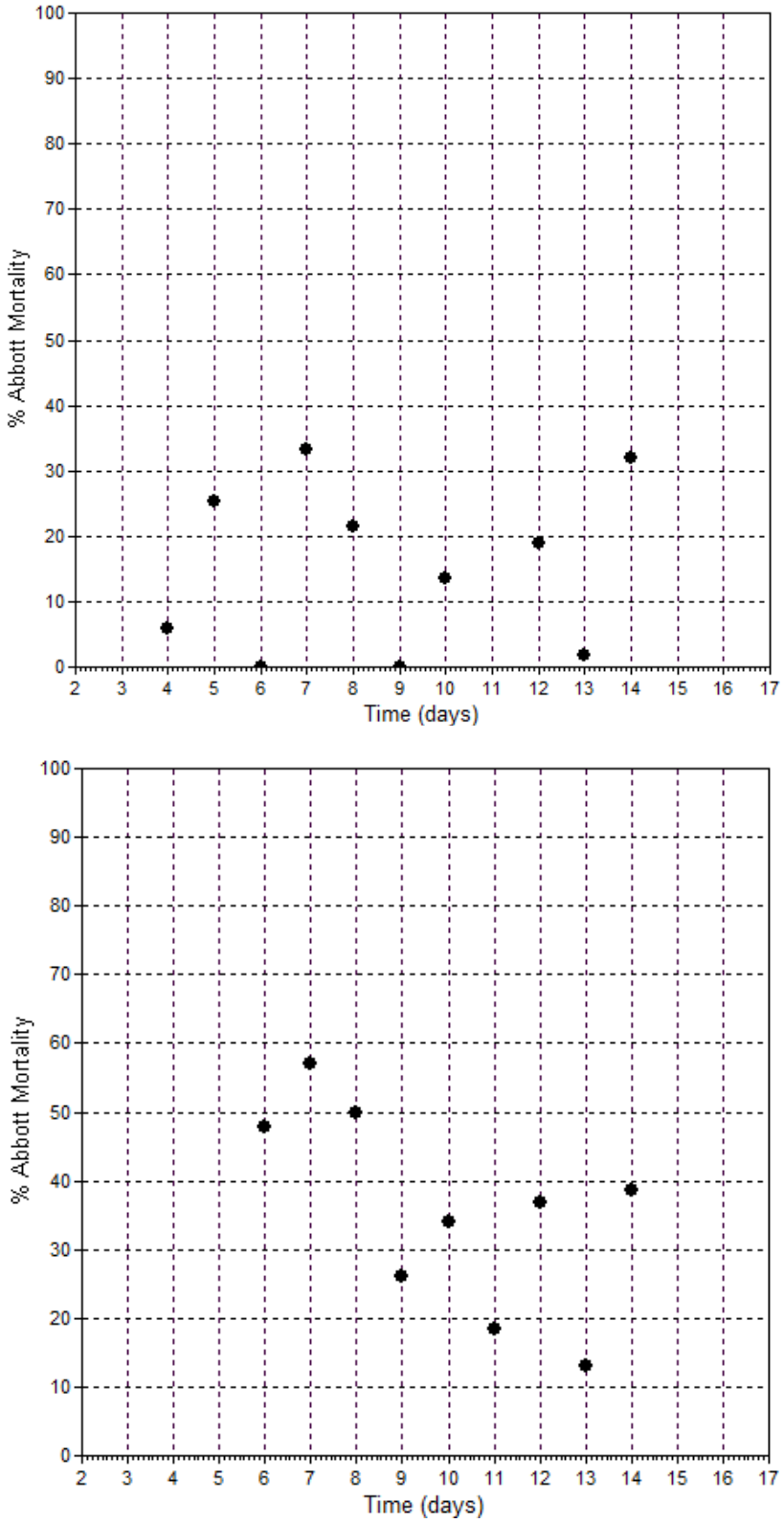

Figure 6. Time/mortality of neonate *P. operculella* larvae treated with isolate PhopGV-IT2.1 (1.1×10^6 OB/ml (LC_{70}) on the upper graph. The lower graph shows the data of a bioassay with the same isolate but a 10 times higher concentration applied (1.1×10^7 OB/ml).

The estimated LC_{80} concentration of isolate PhopGV-IT2.1 did not lead to a significant time/mortality response during 14 days of incubation. Neither with a 10 times higher concentration (1.1×10^7 OB/ml (LC_{80}) no increase of mortality was detectable over the time.

The statistical analysis is shown in Table 2. The LT_{50} value for isolate PhopGV-GR1.1 was calculated at day 10.2 post infection with a LC_{80} concentration of 1.3×10^4 OB/ml inoculum. The application of a lower concentration with a LC_{60} (3.6×10^3 OB/ml inoculum) led to an increased LT_{50} value up to 17 days. The use of a LC_{99} (5.1×10^6 OB/ml inoculum) resulted in 50% mortality at 4.5 dpi.

Isolate IT2.1 showed no time/mortality response and even a negative slope for the LC_{80} concentration (1.1×10^7 OB/ml inoculum) has been observed.

Table 2. Statistical parameters of median lethal time (LT_{50}) values of the tested PhopGV isolates for different virus concentrations against Phop-IT.

PhopGV Isolate	Origin	n Number of Tested Insects	(%) Control Mortality	OB/ml Concentration (Calculated) (LC_{14days})	(%) Mortality at day 14[a]	LT50 (days) (95% Fiducial Limits)	Slope	Chi2 (df)
GR1.1	**Greece** (HELLAFARM)	1840	36.8	3.6×10^3 (LC_{60})	21.81	17.35 (13.1 - 67.2)	0.95	8.47 (7)
		1360	34.4	1.3×10^4 (LC_{80})	77.12	10.21 (9.7 - 10.8)	4.39	12.35 (7)
		640	36.3	5.1×10^6 (LC_{99})	76.5[c]	4.5 (3.0 - 5.9)	1.19	11.48 (6)
IT2.1	**Italy** (BIOGARD)	880	30.6	1.1×10^6 (LC_{70})	31.97	n.d.	(0.42)[b]	45.05 (8)
		1880	36.8	1.1×10^7 (LC_{80})	38.50	n.d.	(-1.95)[b]	48.91 (7)

[a] Abbott (1925) corrected, [b] no meaningful concentration response was found ($p(F) > 0.05$; *i.e.* slope of the relationship is not significant different from zero), [c] value after 11 days. A determination of the LT_{50} for isolate IT2.1 was not possible.

Virus Caused Mortality of a Different *P. operculella* Strain from Tunisia

The most active isolate GR1.1 (based on results with Phop-IT) and a weakly virulent isolate LS1.1 were tested against a Tunisian strain of *P. operculella* (Phop-TN), to investigate the possible influence of different host strains on the PhopGV caused mortality (Table 3).
Neonate larvae were infected with a discriminating concentration (LC_{95}) and mortality was scored at 7 and 14 days post infection. This discriminating concentration was based on bioassay results with a Phop-IT where a mortality of 95% was observed after applying the isolate GR1.1 in a concentration of 1.8×10^5 OB/ml.

Table 3. Mortality values for PhopGV-GR1.1 and PhopGV-LS1.1 against neonates of *P. operculella* originating from Tunisia (Phop-TN) after 7 and 14 days incubation at 28 °C and 16/8 h light/dark photoperiod. Mortality data are based on two independent replicates.

PhopGV Isolate	Origin	N	Mortality 7 Days (%)	Mortality 14 Days (%)
GR1.1	Greece	240/280	45.8	73.0
LS1.1	Unknown	200/280	51.0	71.0
Control		480/480	36.3	46.0

In contrast to the Italian strain of *P. operculella* (Phop-IT) the former weakly virulent isolate LS1.1 caused a similar mortality (51%) as isolate GR1.1 (45.8%) after 7 days post infection with a concentration of 1.8×10^5 OB/ml. No difference in activity was visible after 14 days, when both isolates caused a mortality slightly above 70%. The LC_{95} was not reached for both isolates with a concentration of 1.8×10^5 OB/ml.

Discussion

The majority of the tested PhopGV isolates showed no reasonable mortality after 14 days, this observation confirmed the classification of PhopGV as a slow-killing baculovirus (Gómez Valderrama et al., 2017). PTM larvae infected with PhopGV completed larval development but failed pupation, as described in literature (Sporleder et al., 2005). The larvae were unable to pupate but did not die immediately, instead they stuck in the larval stage till death at a later point of time. Mortality records on day 14 showed clearly the inability of virus-infected larvae to pupate but only few dead individuals were counted at this early point of time, as long as the virus-infected larvae lived on. The infected larvae did not undergo further metamorphosis and were not able to spawn a following generation (Sporleder et al., 2005). Experiments showed that the pupation of control larvae started at day 9, virus-treated individuals were retarded in their developmenent and pupation earliest started on day 11 but on the example of isolate PhopGV-GR1.1 the virus-treated individuals did not exceed a pupation rate of 10% at an applied LC_{80} of virus. Further prohibiting effects on the pupation rate of PTM after PhopGV application could also be observed for other PhopGV isolates (Larem et al., 2018).

The disadvantage of the bioassay test system was that a lack of an artificial diet led to a maximum of 14 days for mortality experiments on fresh potato tuber material, otherwise the diet was too dry to allow further larval feeding or secondary saprophytes, like fungi, occurred when experiments exceeded the duration of two weeks. Three isolates built an exception with visible virus activity on day 14, namely isolates PhopGV-GR1.1, PhopGV-IT2.1 and PhopGV-#1390.2.1. Whereas only PhopGV-GR1.1 reached an LC_{80} on PTM strain Phop-IT and allowed a determination of an LT_{50}. The example of PhopGV-IT2.1 did not show any time mortality correlation. Even applying a 10-times higher concentration did not change the result. This indicated that isolate PhopGV-IT2.1 may act in a different mode of infection compared to PhopGV-GR1.1. Larvae infected with PhopGV-GR1.1 showed an increasing mortality over time. In contrast, PhopGV-IT2.1 infected larvae showed mortality in the first days of infection, with the highest mortality on seven days post infection, but no increased mortality over the time. In contrast to PhopGV-GR1.1, the isolate PhopGV-IT2.1 did not cause a high mortality level during the whole duration of the experiment. Virus susceptibility was host strain dependent, which was observed while LC_{95} of Phop-IT corresponded to a LC_{70} if applied to Phop-TN. Further, highly virulent und low virulent virus isolates against Phop-IT acted with a different result on Phop-TN. Both virus isolates showed virulence on the same medium level against Phop-TN.

Conclusion

The population Phop-IT was susceptible for the tested PhopGV isolates, but only low virus caused mortality was observed until evaluation 14 dpi. The highest activity was obtained with isolate PhopGV-GR1.1 but with a relative low speed of killing (LT_{50} = 10 days). A second *P. operculella* strain Phop-TN showed a different susceptibility against certain PhopGV isolates compared to strain Phop-IT. It was confirmed that PhopGV is a slow-killing virus, which prevents pupation of infected *P. operculella* larvae. Acute heavy infestations with *P. operculella* may be hard to control with this virus but the results of this study indicate the potential of PhopGV as a tool to control *P. operculella* on population level by prohibiting a built-up of following insect generations.

Chapter III

Elucidating the Genetic Diversity of Phthorimaea operculella granulovirus (PhopGV)

Abstract

Twelve complete genome sequences of Phthorimaea operculella granulovirus (PhopGV) isolates from four different continents (Africa, South America, Asia and Europe) were analysed after Illumina Next Generation Sequencing (NGS). The isolates have a circular double stranded DNA genome of 118,355 to 119 kbp in length; all of them encode 130 ORFs. Analysis of single nucleotide polymorphisms (SNPs) revealed a unique set of SNP positions for every single tested isolate. The genome sequences of the investigated PhopGV isolates were classified into a new system of four (1-4) groups according to the presence of group-specific SNPs as well as insertions and deletions. These genome groups correlated with phylogenetic lineages inferred from minimum evolution trees of the whole genome nucleotide sequences. Whereas the geographic origin and the group assignment did not correlate for isolates belonging to the genome groups 1, 2 or 4, members of group 3 showed regional characteristics. Based on high coverage of more than 1,000 reads per nucleotide position, the high resolution of the Illumina NGS data allowed the identification of mixed SNP frequencies, single positions where a SNP but also the reference was supported. In conclusion the geographic isolates of PhopGV are genetically highly similar. On the other hand, they were rarely genetically homogenous and appeared in most cases as mixtures of multiple genotypes.

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Introduction

Phthorimaea operculella granulovirus (PhopGV) is a member of the genus *Betabaculovirus* belonging to the family of *Baculoviridae*. The virus genome consists of circular double stranded DNA with a total length of about 119 kbp encoding 130 open reading frames (ORFs) (Jukes et al., 2016; Croizier et al., 2002). The length of the genome can differ in size among different isolates (Jukes et al., 2016). PhopGV was first isolated from infected potato tuber moth larvae in Sri Lanka (Reed, 1969); it has been found in various parts of the world in correlation with the distribution of its hosts (Espinel-Correal et al., 2010; Zeddam et al., 1999; Kroschel and Koch, 1996; Hunter et al., 1975).

PhopGV has a limited host range to members of the lepidopteran family of Gelechiidae but can infect more than six species within that family, *e.g.* the potato tuber moth (PTM) *Phthorimaea operculella*, Guatemalan potato moth *Tecia solanivora*, tomato leaf miner *Tuta absoluta* (Lacey and Kroschel, 2009), *Symmetrischema tangolias* (Gyen), *Eurysacca quinoae* (Povolny) and *Paraschema detectendum* (Povolny) (Carpio et al., 2013; Povolny, 1967). Most PTM larvae die within 2-3 weeks after ingestion of virus, though very high dosages of PhopGV can cause death by toxicosis within 48 hours (Reed, 1971).

PhopGV has already been used in Latin America e.g. in Colombia, Peru, Costa Rica, Bolivia and Ecuador (Haase et al., 2015) and in Yemen (Kroschel et al., 1996) for control of PTM. In general, PhopGV is a slow-killing granulovirus, and this knowledge is crucial for the correct use as a biocontrol agent (Gómez Valderrama et al., 2017). Infection of PhopGV spreads from midgut to fat body and hypodermis. Hence, only limited parts of host tissue become infected in PhopGV diseased larvae (Lacey et al., 2011). In contrast, Cydia pomonella granulovirus (CpGV) is killing its codling moth host larvae in 5-10 days and is considered as fast-killing granulovirus (Hilton, 2008; Federici, 1997). Here, the fat body is infected along with the epidermis, Malpighian tubules, tracheal matrix, hemocytes, and many other tissues to a lesser extent. Fast-killing granuloviruses (GVs) tend to kill the larvae in the same or following instar in which they become infected, whereas slow-killing GVs retard development of infected larvae and tend to kill the infected host larvae in the final instar, regardless of the stage which was infected (Federici, 1997). Fast-killing baculoviruses have the potential to control pests more efficiently in the short-term than slow-killing ones which may have a higher horizontal transmission efficiency and can control pests effectively in the long-term (Takahashi et al., 2015).

Previously, PhopGV isolates of different geographical origins were characterized by restriction endonuclease (REN) analysis of genomic DNA preparations, with the result of only minor variability among different virus isolates (Carpio et al., 2013; Zeddam et al., 2013; Gómez-Bonilla et al., 2011; Zeddam et al., 1999; Vickers et al., 1991). On the other hand, PhopGV isolates differed in their biological activity against various hosts reported for PTM, *T. absoluta* and *T. solanivora* in laboratory bioassays (Gómez-Valderrama et al., 2017; Zeddam et al., 2013; Espinel-Correal et al., 2010), indicating that there are differences in the genome which cannot be correlated to REN analysis patterns. Nevertheless, genotype mixtures could be identified by analysing submolar bands in DNA REN patterns of PhopGV isolates and were eventually confirmed by PCR and sequencing of particular regions of the viral genome (Zeddam et al., 2013; Espinel-Correal et al., 2010). These sequenced gene regions comprised namely PhopGV ORF 46, repeat region 9 (between PhopGV ORF 83 and 84), the intergenic region between PhopGV ORF 90 and ORF 91, as well as ORF 129. ORF 129 encodes for ecdysteroid UDP–glucosyltransferase *(egt)* which represents a region with the largest genetic variability in the PhopGV genome. Internal insertion and deletion mutations result in an *egt* gene which can appear in five different variations. These variations allowed distinguishing and

grouping of PhopGV isolates into five groups, namely the *egt* types I-V (Jukes et al., 2016; Jukes et al., 2014; Carpio et al., 2013; Zeddam et al., 2013; Espinel-Correal et al., 2010).

So far two whole genome sequences of PhopGV are available at GenBank, with PhopGV-1346 (NC004062) from Tunisia as reference for the species Phthorimaea operculella granulovirus (Espinel-Correal et al., 2010) and a South African isolate PhopGV_SA (KU666536) (Jukes et al., 2016).

For this study, twelve PhopGV isolates were retrieved from laboratory collections as well as from the field. It revealed that many of these PhopGV isolates belong to the same group following the mentioned *egt* typing system. It is important to be able to distinguish between these isolates or to identify samples which are similar to each other. For that reason, whole genome Illumina next generation sequencing (NGS) was carried out for these PhopGV isolates. After analysis of the sequencing data a number of new genetic marker genes for distinguishing different isolates could be identified. Furthermore, the sequenced PhopGV isolates were grouped into four lineages following the differences found in the whole genome nucleotide information.

Material and Methods

Insect Rearing

A rearing of the potato tuber moth *Phthorimaea operculella* was established at the Institute for Biological Control, JKI Darmstadt, in 2014. The insects originated from a laboratory colony isolated in Emilia Romagna, Ravenna, Italy (COOP. TERREMERSE, Bagnacavallo).The larvae were kept on potato slices at 26 °C and under 16/8 h light/dark photoperiod until pupation. Potato slices were placed on sand to allow pupation outside of the potato and to facilitate the collection of the pupae with a mesh. After hatching the adults were transferred to open plastic cylinders (⌀ 24.5 cm, height = 18 cm). The cylinders were lined with a dark plastic bag in order to protect the insects from solar irradiance. The top end of each cylinder was covered with one fine gauze and an additional layer of filter paper. The gauze was permeable for egg laying on the filter paper. This technique allowed an exchange of the egg paper with a fresh one without opening of the cylinder. The adults were fed with 10% sucrose solution. After the collection of the egg paper, the eggs were incubated at either 20 °C or 26 °C, to regulate the hatching day of the neonate larvae. Neonates were used for successive rearing cycles as well as bioassay experiments.

PhopGV Isolates

Different PhopGV isolates (Table 1) were obtained either from infected larvae or as purified occlusion body (OB) suspension. All obtained virus samples were initially propagated in the established laboratory colony of *P. operculella* to obtain a sufficient OB stock of all isolates. Isolates that were propagated in one cycle in *P. operculella* were given the suffix ".1" to their isolates names. If a second round of propagation followed, the suffix ".2" was added (Table 1).

Two of the passaged isolates namely CR3.1 and CR5.1 had their origins in Costa Rica. These two isolates were previously described as CR3 and CR5 and characterized by *egt* sequence analysis and whole DNA REN digests (Zeddam et al., 2013; Gómez-Bonilla et al., 2011). Isolate YM.1 was

collected in Yemen in 1989 which was followed by several publications describing the biological activity and a possible role in the integrated pest management for controlling PTM in Yemen (Kroschel and Koch, 1996; Kroschel, 1995). The isolates GR1.1 and GR2.1 were isolated from soil samples collected from a potato production area in Greece in the framework of the BIOCOMES project in 2014. Isolate IT1.1 derived from a soil sample from tomato production in Italy collected during the same project in 2014. Laboratory strains LS1.1, LS2.1 and LS3.1 were obtained from Horticulture International in Warwick/Wellesbourne. LS1.2 is a production batch of isolate LS1.1 produced by Andermatt Biocontrol (Switzerland). PhopGV-R was purified from the *P. operculella* rearing Phop-IT at the JKI in Darmstadt, this internal virus was described in Larem et al. (2018, submitted).

Virus Propagation

The surface of each potato disc (4.3 cm Ø, 0.5 cm thickness) was inoculated with 200 µl OB suspension (1×10^4 OB/ml). Twenty neonate larvae of *P. operculella* were transferred onto one potato disc using a fine paint brush. The potato discs were kept at 26 °C, 60% RH and 16/8 h light/dark photoperiod. After six days the inoculated larvae showed typical baculovirus infection symptoms like loss of mobility, decreased feeding rate and change in colour from green to bright white followed by sluggishness and flaccidity (Gómes Valderrama et al., 2017; Lacey et al., 2011; Briese 1981; Reed, 1969). From this moment on the potato discs were examined daily and dead larvae were collected.

Purification of Occlusion Bodies from Infected Larvae

Virus OB were isolated from infected larvae according to the protocol of Smith and Crook (1988) with some changes for upscaling from single larvae to pooled larvae samples. To obtain an OB stock for all following experiments approximately 50-60 virus infected insect cadavers were homogenized in 15 ml dH_2O using an Ultra-Turrax (IKA T25, Janke & Kunkel Labortechnik, Staufen, Germany). SDS was added to a final concentration of 0.5%, followed by an incubation for 30 min on ice. Subsequently the suspension was incubated for 3 min in an ultrasonic water bath. Larger larval debris was removed from the suspension by filtration through a double layer of cotton. The filter was washed with 4 ml additional volumes of 0.5% SDS (or 50 mM Tris pH 8.0) and dH_2O, to rinse remaining OBs from the filter into the collection tube. Then the collected suspension was centrifuged for 15 min at 22,000 × g at 12 °C. The supernatant was removed and the pellet was washed two times with dH_2O before resuspending the obtained pellet in 2 ml dH_2O. A discontinuous glycerol gradient 80/70/60/55/50% (v/v) was used to purify the OB. After centrifugation (Eppendorf 5810R) for 45 min at 3,200 × g (swinging bucket rotor) the remaining supernatant and the first three layers of the gradient (50-60%) were collected and washed with water (15 min at 22,000 × g). OBs were resuspended in 2 ml dH_2O and centrifuged for 15 min at 20,800 g. The final OB pellet was then resuspended in 500 µl dH_2O and stored at -20 °C.

OB Quantification

The OB concentration was determined using a Petroff Hausser counting chamber (depth 0.02 mm) (Hausser Scientific, Horsham, Pennsylvania, USA) in the dark field optic of a light microscope (Leica, DMRBE, Leica Microsystems GmbH, Wetzlar, Germany). The concentration was calculated based on the mean of three independent counting steps of an appropriate dilution of each PhopGV isolate. This procedure of virus titration was repeated before the beginning of every experiment with a given isolate.

DNA Extraction

Viral DNA was extracted from OB suspensions by alkaline lysis followed by ethanol chloroform precipitation. For each DNA extraction, about 2×10^{10} OBs of each sample were centrifuged (15 min at 14,000 × g) and a volume of 400 µl of 100 mM Na_2CO_3 (pH > 10) was added to the pellet. The suspension was incubated for 30 min at 37 °C to dissolve the granulin matrix of the OB. After incubation, 40 µl of 1 M HCl was added for pH neutralization before adding 2 µl RNAse A (10 mg/ml) and incubating of 10 min at 37 °C to remove any RNA contamination from the sample. To remove contaminating proteins, 44 µl SDS (10%) and 6 µl Proteinase K (20 mg/ml) were added and incubated for 1 h at 37 °C. DNA isolation was performed by phenol/chlorophorm extraction followed by ethanol precipitation (Arends and Jehle, 2002). The precipitated DNA was pelleted by centrifugation for 15 min at 20,800 × g at room temperature (RT). The pellet was washed with 1 ml 70% EtOH and air-dried until all EtOH has evaporated. The DNA pellet was dissolved in 50 µl dH_2O for further use. Finally, 30 µl DNA solution (> 150 ng) from each isolate were applied for sequencing. DNA concentration was determined by measuring UV absorption of 1 µl DNA sample applied to a Nanodrop 2000c Spectrometer (Thermo Scientific, Wilington, Delaware USA).

Whole Genome Sequencing

Sequencing was performed for all twelve isolates (Table 1) by Illumina NextSeq500 using 150 ng DNA per sample. The DNA library construction was assessed using a NexteraXT/Agilent SureSelect QXT kit with barcoded adaptors for multiplexing generated read inserts sized between 200 and 800 bp. The DNA libraries were sequenced by paired-end option and produced reads of 150 bp (StarSEQ GmbH, Mainz, Germany). The following bioinformatics was performed using the JKI Galaxy Server and the genome analysis program Geneious 10.0.5 (Biomatters Ltd, Auckland, New Zealand). The genome sequence of isolate PhopGV-1346 (NC004062) was used as reference. This reference isolate with origin in Tunisia was the first fully sequenced PhopGV isolate published on GenBank. Later the genome sequence of the South African isolate PhopGV_SA (KU666536) became available (Jukes, 2016).

Bioinformatic Analysis of Whole Genome Data

CLC Assembly and Bowtie2 Analysis

Illumina sequencing output provided more than 800,000 forward and reverse sequences of 150 bp length per sample. The assembly of all forward reads, was followed by the concatenation with all assembled reverse reads. Sequencing reads were additionally trimmed using the Galaxy Server tool "Trim Galore" removing residual adaptor sequences and such of low base-call quality. All reads shorter than 76 bp after trimming were discarded. CLC *de novo* assembly of each analysed PhopGV isolate and a Bowtie 2 mapping against the reference genome (PhopGV-1346) were carried out in parallel with the obtained trimmed sequences (Figure 1). For the CLC procedure the paired reads were assembled with a minimum distance of 200 nt and maximum distance of 800 nt. The reads were assembled to contigs with a minimum length of 200 nt. As additional parameters of the CLC assembly a word size of 64 and a bubble size of 400 were applied. These two values were set high to allow covering large insertions and deletions (Indels). The output of the CLC assembly was a *de novo* consensus of the reads. By analysing the Bowtie 2 data there was a risk to achieve misleading information coming from the reference isolate, especially at low coverage sites. The reads of an isolate were mapped to the reference genome with the adjustment "very sensitive local" and the maximum fragment length for the valid paired-end alignment was set to 500. The independent methods: the *de novo* assembly (CLC) and mapping to the reference (Bowtie 2) were used for every sequencing dataset to avoid ambiguities in the final consensus sequence of each newly sequenced PhopGV isolate.

Additional *De Novo* Assembly

The two applied methods CLC Assembly and Bowtie2 analysis provided each one consensus sequence (Figure 1). A whole genome alignment of these two consensus sequences was leading to ambiguities in some cases. To eliminate these ambiguities, a second *de novo* assembly was performed in parallel for each sequencing sample in order to minimize errors, which could have occurred by the weakness of one single analysing method. This second assembly was performed on all trimmed and quality filtered (cut off 30, *p*-value 90) reads using the *de novo* assembly function in Geneious 10.0.5 software (Biomatters Ltd, Auckland, NZ) with medium-low sensitivity options. This third method helped to make a clear decision for ambiguity locations when differing CLC and Bowtie2 information occurred.

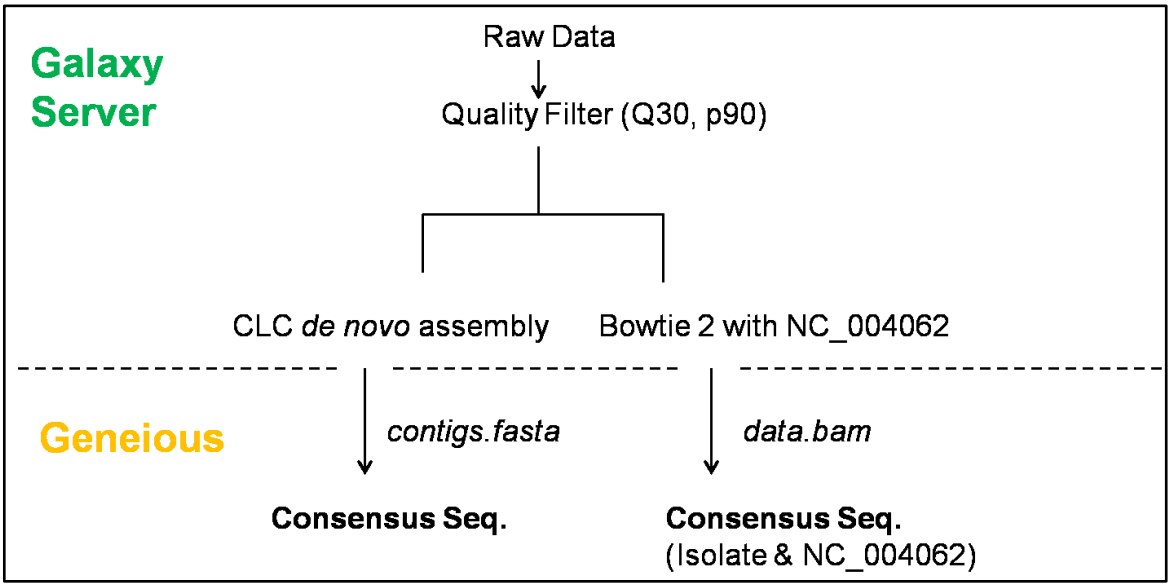

Figure 1. Overview of genome bioinformatic analysis to obtain NGS consensus sequence using two independent platforms.

Identification of Indel Mutations

Insertions and deletions (Indel mutations) were identified by whole genome alignment of all sequenced isolates against the reference PhopGV-1346 (NC004062) using MUSCLE 3.6 with the Mauve aligner algorithm (Darling et al., 2004), a plugin developed for Geneious 10.0.5. Indel mutations occurring inside of a coding region were analysed by translation of the respective ORF to its amino acid sequence and alignment against the reference amino acid sequence of PhopGV-1346 with ClustalW Alignment of Geneious 10.0.5.

SNP Detection

The occurring single nucleotide polymorphisms (SNPs) for each sequenced isolate were identified using the SNP calling tool MPileup of the JKI Galaxy Server. All sequencing data of the twelve isolates were handled in the same manner to secure an equal analysis of each dataset allowing a reliable comparison to each other. Every SNP position refers to the reference PhopGV-1346 (NC004062). The SNP frequencies were calculated using the number of reads supporting a nucleotide variant and those supporting the reference nucleotide. Some reads showed ambiguities because of sequencing errors and were filtered out by quality. All reads that did pass the quality filtering and mapping process were considered as source of information for single genome positions. If all reads were supporting the reference the SNP frequency value was set to 0 and no SNP was considered for that position. If all reads were supporting an alternative the value 1 was set and the SNP was considered to be homogeneous. A value between 0 and 1 indicated a nucleotide variability at a given position.

Phylogenetic Analysis

The generated consensus sequences of the PhopGV isolates were used for a whole genome nucleotide alignment using MUSCLE 3.6 with the Mauve aligner algorithm (Darling et al., 2004) of the program Geneious 10.0.5. The alignment was exported and adapted with the program BioEdit version 7.2.5 in order to insert gaps at the end of the sequences to bring the sequences to the same length for the calculation of phylogeny with MEGA6 (Tamura et al., 2013). This adaptation was necessary for the outgroup definition. For analysing the relationship among the different PhopGV isolates the minimum evolution method with Kimura-2 parameters (Kimura, 1980) and 500 bootstrap replicates were used for the calculation of the phylogenetic tree. Pairwise deletions were recognized as informative sites.

PCR Amplification

For the amplification of the intergenic region between ORF 109 (*late expression factor 9, lef-9*) and ORF 110 (*fusion protein, fp*) a specific oligonucleotide primer pair has been used, namely orf109/110F (5'-GCC AAC ACC AAA GAG GAG GA-3') and orf109/110R (5'-CGC CGA ACA CCT CTA CAC AT-3') based on PhopGV-GR1.1 nucleotide sequence. The primer pairs were flanking the intergenic region by binding inside the ORFs 109 and 110. Reactions comprised 5 µl 10x detergent free reaction buffer (Axon Labortechnik, Kaiserslautern, Germany), 2 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTPs, 2 µl primer forward and reverse (10mM), 50 ng DNA template and 0.5 µl taq polymerase (Axon Labortechnik GmbH, Kaiserslautern, Germany). Reactions were made up to 50 µl using ddH_2O and the contents were briefly centrifugated. The PCR protocol was: 94 °C for 4 min, followed by 33 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min. Amplified products were visualised by 1% agarose gel electrophoresis at 90 V for 45 min in 1x TAE buffer (40 mM Tris-acetate, 20 mM acetic acid, 1mM EDTA) stained with Midori Green Advance (Nippon Genetics Europe GmbH, Düren, Germany). Visualisation and caption of the gel images was done with ChemoCam Imager ECL UV trans-illuminator and software (INTAS Science Imaging Instruments GmbH, Göttingen, Germany).

Results

Twelve PhopGV isolates were analysed for this study and an overview of all sequenced PhopGV isolates as well as general genome information is given in Table 1. Between 854,690 and 1,647,866 reads resulting in a minimum average coverage of 1,079 reads per nucleotide position were obtained for the different isolates. After quality filtering of the raw data and the removal of unmapped reads the average coverage per nucleotide (nt) was above 1,000 reads for each isolate. All isolates had the same number of 130 identified ORFs similar to the reference isolate PhopGV-1346 (NC004062) and PhopGV_SA (KU666536) indicating a low coding heterogeneity among the isolates. Genome annotation is given in SupplementaryTable S III 1 for all PhopGV isolates. The GC content of 35.7% was stable for all isolates. Differences were found in the total genome sequence length of the twelve isolates, which ranged from 118,355 bp (PhopGV-CR3.1) to 119,177 bp (PhopGV-IT1.1). The differences in length were attributed to indels dispersed throughout the genome sequences. Large Indels were present in ORF 129 (*egt*), the size differed 267 nt between the analysed PhopGV

isolates. In addition, there was an insertion of 150 nt between the ORF 109 (*late expression factor 9, lef-9)* and ORF 110 (*fusion protein, fp*) for four isolates (Gr1.1, GR1.2, GR2.1 and IT1.1) compared to the reference PhopGV-1346 and the remaining analysed PhopGV isolates. A deletion of 45 nt occurred in ORF 104 (*desmoplakin*) and as well for 27 nt in ORF 46 for two isolates (YM.1, LS2.1). ORF 33 (*ODV-e66*) had a deletion of 24 nt for five isolates (LS1.1, LS1.2, LS3.1, CR3.1 and CR5.1).

SNP Distribution

A total number of 503 SNP positions were identified according to the PhopGV reference genome. The majority of 313 SNPs clustered within the first 60,000 nt of the reference genome, in which genome annotation and nucleotide numbering was orientated to *granulin* (*orf 1*), whereas 190 SNP positions spread over the second half of the genome (> 60,000 nt) (Figure 2). Hot spots for SNP clustering occurred around nucleotide position 6,000 (17 SNPs), between 10,000 and 15,000 (43 SNPs), between 17,000 and 19,000 (13 SNPs) and around 23,000 (22 SNPs), 26,000 (9 SNPs), 28,000 (11 SNPs), 42,000 (17 SNPs) and nucleotide position 45,000 (14 SNPs). In addition, three genome regions around nucleotide position 77,000 (15 SNPs), 87,000 (9 SNPs) and 111,000 (13 SNPs) with increased number of SNPs were observed. The pattern of the SNP clustering suggested that some isolates were closely related. Distinct patterns could be identified for LS1.1, LS1.2, LS3.1, CR3.1 and R (SNP group 1), LS2.1 and YM.1 (SNP group 2) and GR1.1, GR1.2, GR2 and IT1.1 (SNP group 3). Isolate CR5.1 did not fit into one of the three groups and was assigned to an extra SNP group 4. Also the frequency of the group specific SNPs was more dominant in the first half of the linearized genome than in the second half.

Table 1. General information about the sequenced PhopGV isolates.

Isolate (PhopGV-)	Origin	Reads Assembled	Coverage / nt	Consensus Seq. Length	Accession No.	Source/ Reference
YM.1	Yemen	964,846	1,218	118,936	MK033576	Kroschel 1989
LS1.1	Unknown	1,237,642	1,558	119,157	MK033571	HRI (ID238)
LS1.2	Unknown	854,690	1,079	119,047	MK033572	ABC (ID238)
LS2.1	Unknown	1,228,974	1,549	118,863	MK033573	HRI (ID243)
LS3.1	Unknown	860,723	1,083	119,102	MK033574	HRI (ID244)
GR1.1	Greece	1,647,866	2,076	119,061	MK033567	Hellafarm (ID320), Larem et al., 2018
GR1.2	Greece	986,675	1,243	119,061	MK033568	ABC (ID320)
GR2.1	Greece	1,098,654	1,383	119,139	MK033569	Hellafarm (ID323)
IT1.1	Italy	1,237,642	1,558	119,177	MK033570	Biogard (ID328)
CR3.1	Costa Rica	936,612	1,187	118,355	MK033565	UPNA / Gómez-Bonilla 2011, Zeddam 2013, Larem et al., 2018
CR5.1	Costa Rica	1,244,015	1,570	119,095	MK033566	UPNA / Gómez-Bonilla 2011, Zeddam 2013
R	Darmstadt	1,163,983	1,284	119,080	MK033575	Larem et al., 2018
1346	Tunisia	-	-	119,003	NC004062	Crozier (unpublished)
SA	South Africa	-	-	119,004	KU666536	Jukes 2016

HRI = HRI Collection, Warwick; ABC = Andermatt Biocontrol AG, Grossdietwil, Switzerland; Hellafarm = Hellafarm SA, Attica, Greece; Biogard = Biogard, Division of CBC Europe, Grassobbio, Italy; UPNA = Public University of Navarre, Agrobiotechnology Institute, Pamplona, JKI = Julius Kühn-Institut, Darmstadt, Germany

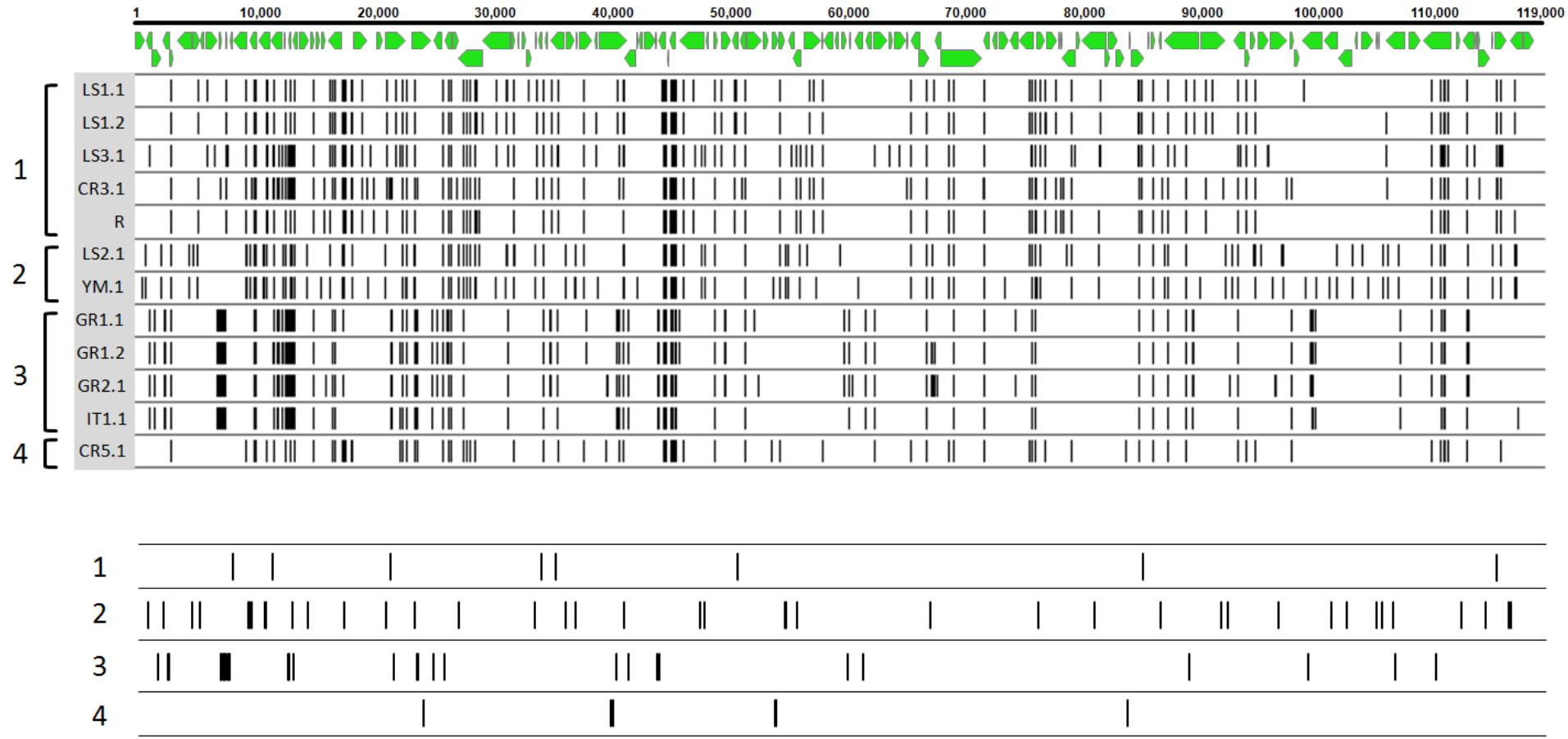

Figure 2. Group specific SNPs of the sequenced PhopGV isolates compared to the reference PhopGV-1346 (NC004062). Top: The genome is shown using green arrows representing the 130 ORFs. The top black bar line indicates the scale from 1 to 119,000 nt. The vertical black lines indicate the SNP positions of each isolate on the genome. Bottom: distribution of SNPs specific to genome groups 1-4. Vertical lines indicate position of those SNPs which are unique to one of the genome groups 1-4.

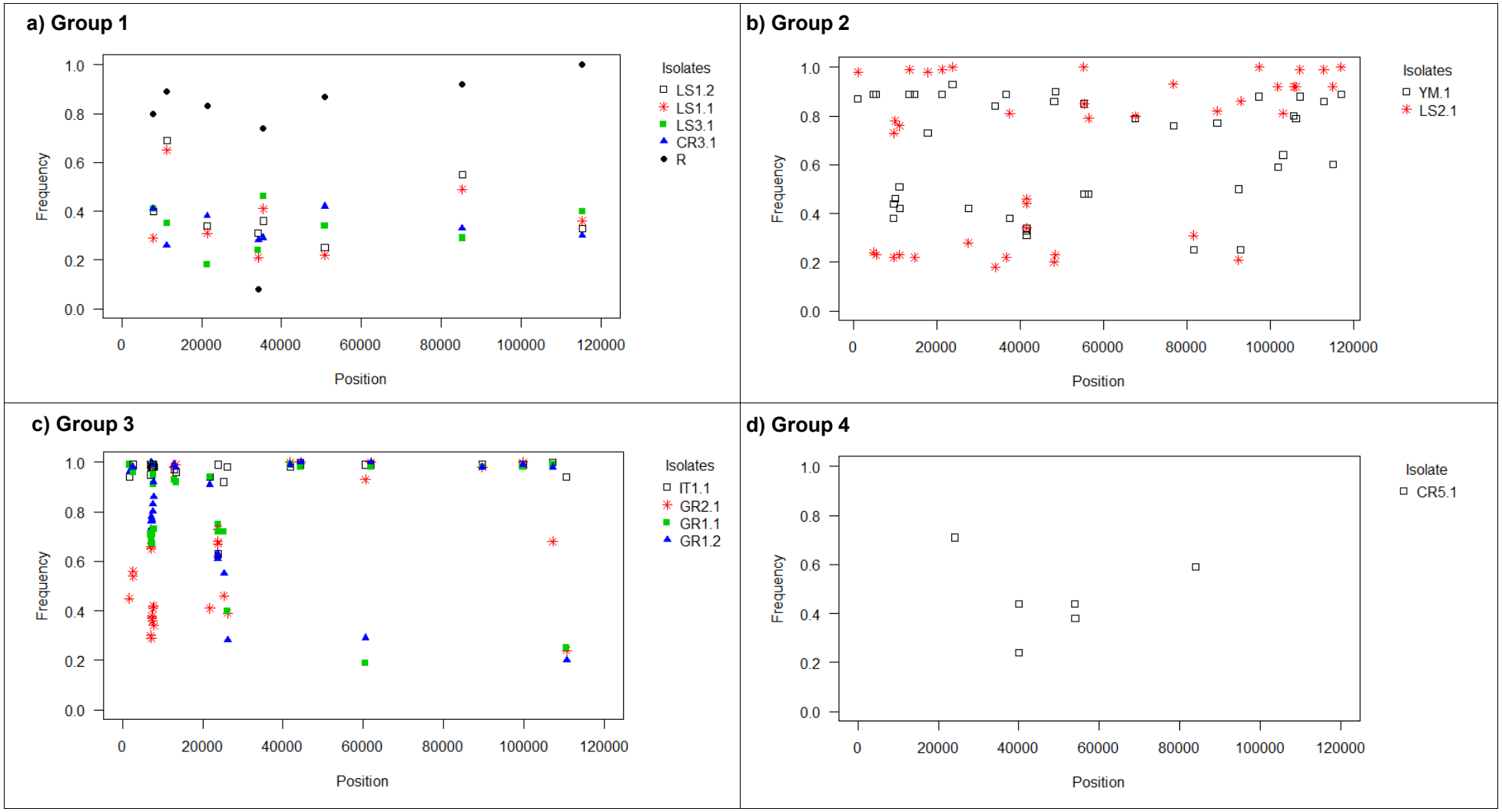

Figure 3. Distribution of group specific SNP frequencies. Given are the frequencies of the four genome groups a) group 1, b) group 2, c) group 3 and d) group 4. Different colours and symbols are used for single isolates as indicated to the right. The positions on the x-axis is relative to the reference isolate PhopGV-1346.

As shown in Figure 2 and Table 2, the number of SNPs per genome ranged between 83 for CR5.1 and 140 for LS3.1. Due to the observation of shared SNPs, the isolates were grouped into four different genome groups (1-4). All isolates of SNP group 1 shared eight SNP positions which are found in no other isolate of the remaining three groups. Thus, these eight SNP positions are group specific for the five isolates LS1.1, LS1.2, LS3.1, CR3.1, and R of SNP group 1. The highest number of group-specific SNPs with 42 SNPs was found for genome group 2, including LS2.1 and YM.1. Isolates of genome group 3 shared 32 SNPs, whereas CR5.1 of genome group 4 had six unique SNPs. The isolates LS1.1/LS1.2 and GR1.1/GR1.2 built an exception because they were passages from single isolates, namely LS1 and GR1, respectively. LS1.1 and LS1.2 shared eight SNPs, which were unique to both isolates but differed from each other in five SNPs unique to LS1.1 and in three SNPs unique to LS1.2 (Table 2). In contrast, GR1.1 and GR1.2 shared the same number of five SNPs unique to both passages. Lowest number of unique SNPs (6) were identified in isolate CR5.1, whereas YM.1 had the highest number of SNPs (28) of all analysed isolates. A detailed Table of SNP positions of the analysed PhopGV isolates is given in the Supplementary Table S III 2.

Table 2. Number of group-specific and unique SNPs compared to the reference PhopGV-1346.

Genome Group	Isolate (PhopGV-)	No. of SNPs	SNPs Shared Group Specific	Unique SNPs
1	LS1.1	110	8	8+5[a]
	LS1.2	115		8+3[a]
	LS3.1	140		24
	CR3.1	135		24
	R	97		-
2	LS2.1	123	42	12
	YM.1	135		28
3	GR2.1	138	32	12
	IT1.1	111		1
	GR1.1	127		5[b]
	GR1.2	129		5[b]
4	CR5.1	83	6[c]	6

[a]Eight SNPs are identical for LS1.1 and LS1.2 but five (LS1.1) and three (LS1.2) SNPs were identified additionally, [b]SNPs are identical; [c]group-specific and unique SNPs are identical

SNP Frequencies

Comparing all genomes a total of 503 SNP positions was found. Whereas at some SNP positions a frequency of 100% was observed for a given nucleotide, other positions harbored mixtures of two nucleotides. However, for all SNP positions no more than one possible alternative nucleotide was observed; presence of a second or third alternative nucleotide was never detected. Analysis of the frequency of SNPs allowed gaining additional information about the genetic composition of a given PhopGV isolate (Figure 2). At some polymorphic sites the variant nucleotide frequencies identified in the different genomes ranged from 0.08 to 1.0. Frequencies at different SNP positions did not show a wide degree of variance within some genomes *e.g.* PhopGV-IT1.1, whereas other isolates, *e.g.* PhopGV-GR2.1, revealed considerable frequency variations indicating a distinct intra-population heterogeneity (Figure 4, Supplementary Table S III 3, Supplementary Table S III 4). In summary, frequencies of the alternative nucleotides of the eight SNPs specific to group 1 were always below 0.7

with exception of the internal isolate R (Figure 4a). Isolate LS2.1 of genome group 2 showed alternative SNP frequencies of > 0.95 in ten out of 42 positions, whereas 16 SNP positions were present with a frequency scattered around 0.8 and another 16 positions occurred with a frequency below 0.5. YM.1 from the same group 2 had very different frequencies of SNPs compared to LS2.1 indicating the difference of the two isolates (Figure 4b). For the 32 specific SNP positions of genome group 3, a high variability was found for the isolates IT1.1, GR1.1, GR1.2 and GR2.1 (Figure 4c). Whereas IT1.1 showed a very high frequency between 0.9 at all positions, the isolates GR1.1 and GR1.2, which represent two passages of the same isolate GR1, were significantly different form IT1.1 at single positions but highly similar to each other. In contrast GR2.1 showed stongest deviations from the other isolates of group 3. CR5.1 (genome group 4) had six specific SNPs with intra-isolate alternative frequencies between 0.3 and 0.7 (Figure 4d).

Indel Mutations

Insertions and deletions (Indel) mutations found in the different isolates were identified to be consistent with the above developed genome grouping system based on SNP distribution. These Indel mutations were present in all isolates of one genome group and absent in isolates of any other genome group; they occurred either within or outside of ORFs. One example for an insertion in a non-coding region is the locus between *lef*-9 (*orf*109) and *fp* (*orf*110), which was unequivocally correlated to isolates of genome group 3. Due to the insertion size of 150 nt it can be used as a group marker to discriminate a member of group 3 to isolates of all other groups (Figure 4).

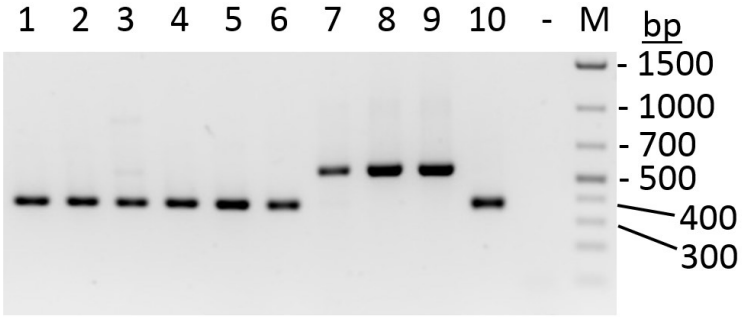

Figure 4. Agarose gel (1%) electrophoresis of PCR products of intergenic region between ORF 109 and ORF 110. Lane 1 to 10 show the PCR products of different PhopGV isolates, lane 1 = LS1.1, 2 = LS3.1, 3 = CR3.1, 4 = R, 5 =LS2.1, 6 = Ym.1, 7 =GR1.1, 8 = GR2.1, 9 = IT1.1, and 10 = CR5.1. Lane 1-4 represent genome group 1, lane 5-6 group 2, lane 7-9 group 3 and lane 10 represents group 4. Negative control (-) was PCR mastermix with dH_2O instead of DNA. Gene Ruler 1 kb plus DNA ladder from Thermo Fisher was used as size marker (M). The gel image was inverted for better visualization of the PCR fragments.

Indel mutations identified inside ORFs were annotated in reference to isolate PhopGV-1346. For isolates of genome group 1 Indels were identified in five ORFs, ORF 24 (*pe-38*), ORF 28, ORF 33 (*odv-e66*), ORF 43 and ORF 129 (*egt*) (Figure 5). ORF 24 (*pe-38*), ORF 28 and ORF 43 showed Indels specific for genome group 1, no other isolate of the other genome groups showed an occurrence of these mutations. The Indels in ORF 33 (*odv-e66*) and ORF 129 (*egt*) were shared with those of group 4 and group 2, respectively. Two deletions of 2 and 4 nt in ORF 129 of genome group

1 caused a deletion of two amino acids (ΔQV) and a frame shift compared to the reference sequence. As a result ORF129 of genome group 1 and group 2 is 16 amino acids longer than that of the reference sequence of PhopGV-1346. The other deletions found for group 1 isolates did not cause a frame shift and resulted in a shorter predicted ORFs compared to the reference PhopGV-1346. ORF 24 was one amino acid (ΔA) shorter, ORF 33 and 43 were shorter in two amino acids (ΔTP and ΔPT), respectively, and ORF 28 showed a deletion of five amino acids (ΔGG and ΔAKK).

For isolates of genome group 2, six Indel mutations were identified. Beyond the insertion in ORF 129 (*egt*) shared with group 1 the remaining five Indels were identified in three hypothetical ORF 32, ORF 46 and ORF 128 as well as the homologues of *vp91* (ORF 94) and *desmoplakin* (ORF 104). The deletions in the ORFs ranged from one amino acid (ORF 32) to 14 amino acids (ORF 104), whereas ORF 94 contained an insertion of six amino acid residues (Figure 5).

Specific Indels of isolates belonging to genome group 3 occurred in ORF 18, ORF 33 (*odv-e66*) and ORF 129 (*egt*). ORF 18 showed an insertion of a single amino acid (+I) and two deletions (ΔFN and ΔC). ORF 33 (*odv-e66*) had an insertion of two amino acids (+PQ). ORF 129 (*egt*) had a deletion of 2 bp causing a frameshift mutation at the 3´ end of the ORF which results in a truncation of predicted 73 amino acids compared to in that of the reference PhopGV-1346 (Figure 5). The two deletions in isolate CR5.1 (genome group 4) were accompanied with predicted amino acid deletions (ΔTP and ΔQPQPAP) in ORF 33 (*odv-e66*). These mutations were not unique to genome group 4 but shared with genome group 1. In contrast to the other isolates the predicted amino acid sequence of ORF 129 (*egt*) was identical to that of the reference (Figure 5).

The resulting lengths of the ORFs specific for the genome groups 1 to 4 could be explained by the sum of Indel mutations and frameshifts caused by these mutations. Only ORF 129 (*egt*) was a special case because the presence of single SNPs generated additional stop codons. The reference PhopGV-1346 had an A at nt 1,311, which generated a stop codon TGA resulting in an ORF of 434 amino acids. In contrast, isolates of genome group 1 and 2 had a G at this position and the resulting codon TGG coded for the amino acid tryptophan (W), extending the ORF 129 (*egt*) to 450 amino acids till the next stop codon was reached. Genome group 3 had a deletion of two bp in ORF 129 (*egt*) causing a frame shift and two SNPs on nucleotide position 1,087 and 1,088 relative to the reference with two A residues forming a stop codon TAA, resulting in an ORF length of 361 amino acids.

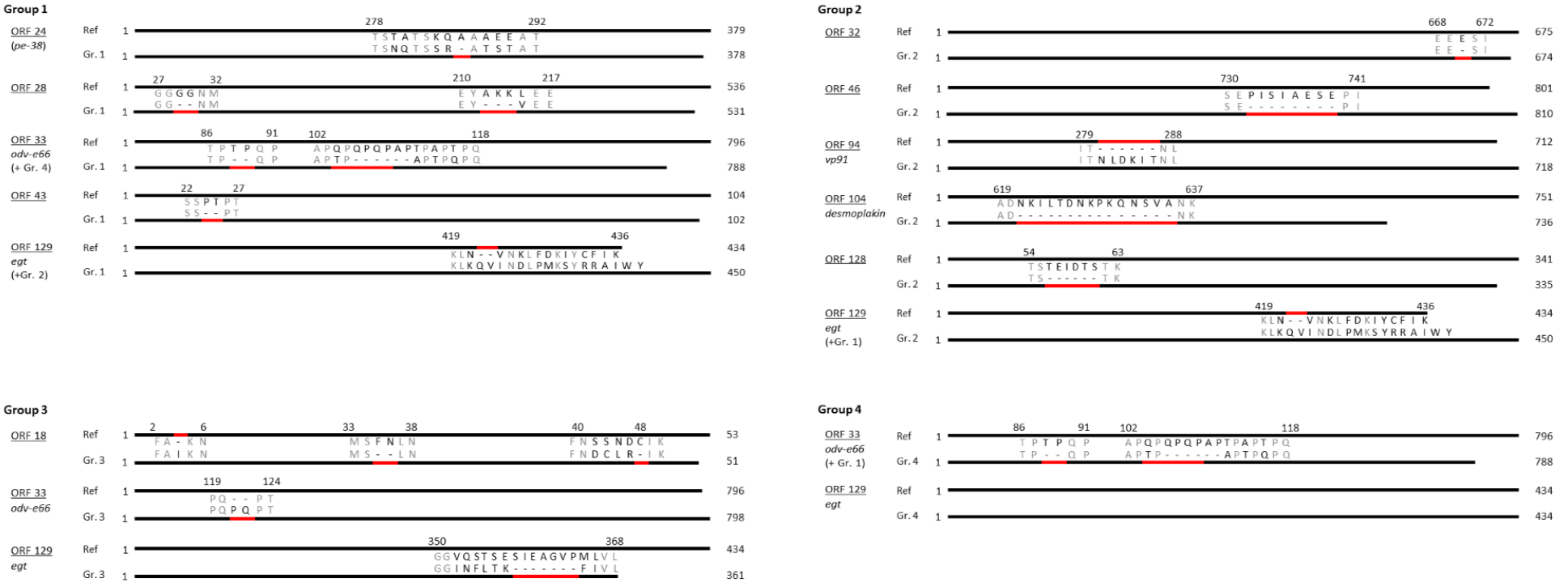

Figure 5. Diagram of amino acid sequences depicting ORFs with Indel mutations. Genome groups (Gr.) 1 to 4 were aligned with the respective ORFs from the reference (Ref) isolate PhopGV-1346 (NC004062). The total protein length of each ORF is shown to the right, while the numbers above each insertion/deletion region represent the corresponding amino acids from the aligned reference sequence. Deletions/insertions are indicated by a red bar.

SOD Types

PhopGV ORF 54 encodes a homologue of the superoxide dismutase (*sod*). The genome comparisons revealed that *sod* was present in different ORF lengths as a consequence of Indel mutations and SNP distributions (Table 3). Again, the occurrence of different *sod* types correlated with the grouping system based on SNP and Indel information: the longest variant of *sod* had a size of 390 bp and was characteristic for isolates of the genome groups 3 and 4, where it appeared in a frequency of 0.93 to 1.0. In YM.1 and IT1.1 (genome group 2), a single deletion (ΔC) in codon 368 caused a frameshift mutation which resulted in an additional stop codon and thus in an 18 bp truncation of the ORF to a total length of 372 bp. For isolate PhopGV_SA a point mutation (C>T) at codon position 211 generated an ochre stop codon and reduced the ORF length to 213 bp. A single nucleotide deletion (ΔG) at position 63 caused a frameshift and a stop codon downstream at nucleotide position 78, resulting in a very short and probably not functional *sod* ORF; this *sod* form was detected in a sub-population of group 1 isolates at frequency of 0.09 to 0.87, as well as in GR1.1 (0.01) and CR5.1 (0.17) (Table 3).

Table 3. Frequency of different variants of ORF 54 (*sod*) of PhopGV isolates. Given are the genome groups of the majority as it is present in the consensus sequence of the isolates, as well as the frequency of different sod types.

Genome Group	**Isolate** (PhopGV-)	**Frequency of *sod* types**			
		78 bp	**213 bp**	**372 bp**	**390 bp**
1	LS1.1	0.87	-	-	0.13
	LS1.2	0.81	-	-	0.19
	LS3.1	0.73			0.27
	CR3.1	0.15	-	-	0.85
	R	0.09	-	-	0.91
2	LS2.1	-	-	1	-
	YM.1	-	-	1	-
3	GR2.1	-	-	-	1
	IT.1.1	-	-	-	1
	GR1.1	0.01	-	-	0.99
	GR1.2	-	-	-	1
4	CR5.1	0.17	-	-	0.83
	1346*	-	-	-	1**
-	SA	-	1**	-	-

*corrected reference (Δ10bp) else *sod* 501 bp length
**no raw read information for reference isolates PhopGV-1346 and PhopGV-SA
For position of ORFs see Supplementary Table III 1.

An *in silico* correction was done for the sequence of the reference isolate PhopGV-1346 to mend a potential sequencing error which had most likely occurred by an insertion of 10 bp resulting in a frame shift and an elongation of the ORF to 501 bp in the original sequence. Deleting these 10 bp resulted in a *sod* ORF of 390 bp, similar to other group 4 and group 3 isolates.

Phylogenetic Analysis

For each isolate a nucleotide consensus sequence was assembled using the majority information of reads of at least > 50%. A minimum evolution phylogenetic tree was inferred using the alignment of the nucleotide consensus sequences of 14 PhopGV genomes (Figure 6). When the taxa were assigned to *egt* types (I, II and III) and the SNP grouping system a considerable correlation with the tree topology could be observed. The *egt* type I was represented two times with PhopGV-1346 and PhopGV-CR5.1. Both isolates were clustered together in the phylogenetic tree and are also considered as SNP group 4. PhopGV-GR1.1, PhopGV-GR1.2, PhopGV-GR2.1 and PhopGV-IR1.1 formed a single clade representing *egt* type III and SNP group 3. The largest number of isolates was assigned to *egt* group II containing the eight isolates PhopGV-LS1.1, PhopGV-LS1.2, PhopGV-LS3.1, PhopGV-R, PhopGV-CR3.1, PhopGV_SA, PhopGV-LS2.1 and PhopGV-Ym.1. When whole genome SNPs and Indels were considered, the *egt* group was divided into three groups, *i.e.* group 1, group 2, and PhopGV_SA which did not fit in any group.

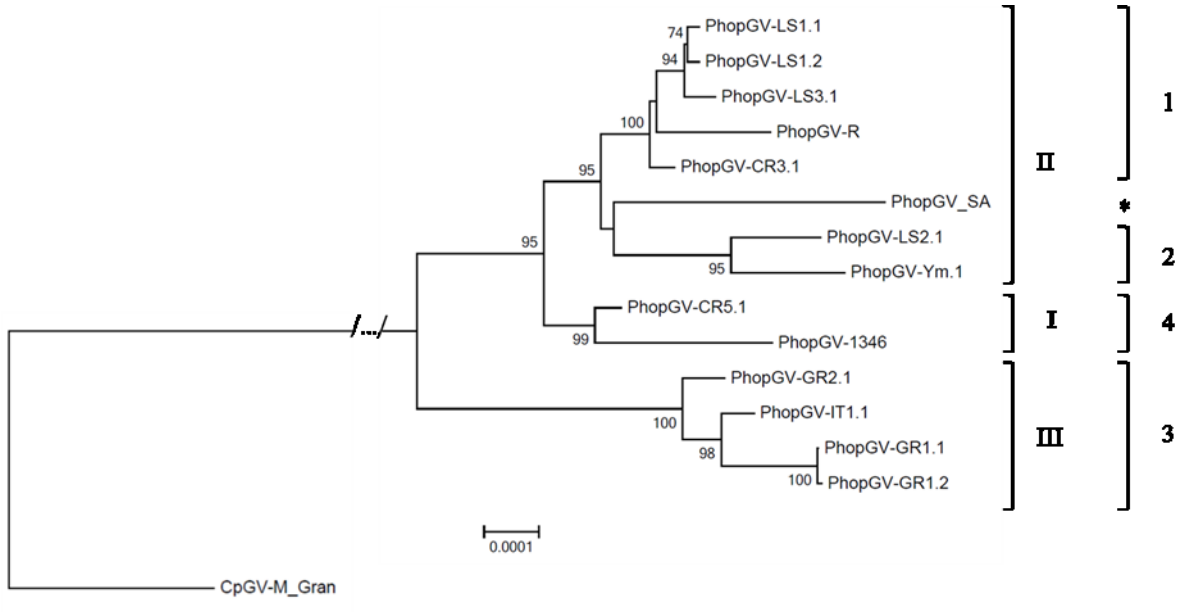

Figure 6. Minimum evolution tree based on whole genome nucleotide information using the method with Kimura 2-parameter distances and a bootstrap value of 500 replicates. The Roman numbers on the right indicate the *egt* type (Jukes et al., 2014), Arabic numbers display the isolate group, the asterisk (*) indicates isolates which did not fit to any group. The tree was rooted by using the *granulin* gene of CpGV-M as an outgroup (Figure 6). The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky and Nei, 1992). The optimal tree with the sum of branch length = 0.19944 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 1. The Neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree. All ambiguous positions were removed for each sequence pair. There were a total of 120,988 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Estimates of Evolutionary Divergence between Sequences

Table 4. Pairwise distances of the concatenated polh(*gran)*/*lef-8*/*lef-9* fragments of the PhopGV/CpGV cluster. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Kimura 2-parameter model (Kimura, 1980). The K-2-P analysis involved 19 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 4,899 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). The genome identity based on the alignment information is displayed deposited in gray.

gran/lef-8/lef-9	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1 PhopGV-LS1.1		**1.000**	**1.000**	**1.000**	**1.000**	**0.999**	**0.999**	**0.999**	**1.000**	**0.999**	**0.999**	**0.999**	**0.999**	**0.999**	**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
2 PhopGV-LS1.2	**0.000**		**1.000**	**1.000**	**1.000**	**0.999**	**0.999**	**0.999**	**1.000**	**0.999**	**0.999**	**0.999**	**0.999**	**0.999**	**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
3 PhopGV-LS3.1	**0.000**	**0.000**		**1.000**	**1.000**	**0.999**	**0.999**	**0.999**	**1.000**	**0.999**	**0.999**	**0.999**	**0.999**	**0.999**	**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
4 PhopGV-R	**0.000**	**0.000**	**0.000**		**1.000**	**0.999**	**0.999**	**0.999**	**1.000**	**0.999**	**0.999**	**0.999**	**0.999**	**0.999**	**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
5 PhopGV-CR3.1	**0.000**	**0.000**	**0.000**	**0.000**		**0.999**	**0.999**	**0.999**	**1.000**	**0.999**	**0.999**	**0.999**	**0.999**	**0.999**	**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
6 PhopGV-SA	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		**0.999**	**0.999**	**1.000**	**0.999**	**0.999**	**0.999**	**0.999**	**0.999**	**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
7 PhopGV-LS2.1	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		**1.000**	**1.000**	**0.999**	**0.999**	**0.999**	**0.999**	**0.999**	**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
8 PhopGV-Ym.1	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.000**		**1.000**	**0.999**	**0.999**	**0.999**	**0.999**	**0.999**	**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
9 PhopGV-CR5.1	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**		**0.999**	**0.999**	**0.999**	**1.000**	**1.000**	**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
10 PhopGV-1346	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		**0.999**	**0.999**	**1.000**	**1.000**	**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
11 PhopGV-GR2.1	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		**0.999**	**0.999**	**0.999**	**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
12 PhopGV-IT1.1	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**		**1.000**	**1.000**	**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
13 PhopGV-GR1.1	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.000**	**0.000**	**0.001**	**0.000**		**1.000**	**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
14 PhopGV-GR1.2	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.000**	**0.000**	**0.001**	**0.000**	**0.000**		**0.697**	**0.697**	**0.697**	**0.697**	**0.697**
15 CpGV-M	0.350	0.350	0.350	0.350	0.350	0.351	0.351	0.350	0.351	0.351	0.350	0.350	0.350	0.350		**0.999**	**0.999**	**0.998**	**0.997**
16 CpGV-I12	0.350	0.350	0.350	0.350	0.350	0.351	0.351	0.350	0.351	0.351	0.350	0.350	0.350	0.350	***0.001***		**0.998**	**0.997**	**0.996**
17 CpGV-S	0.350	0.350	0.350	0.350	0.350	0.351	0.350	0.350	0.350	0.350	0.350	0.349	0.350	0.350	***0.001***	***0.002***		**0.998**	**0.996**
18 CpGV-E2	0.350	0.350	0.350	0.350	0.350	0.351	0.350	0.350	0.350	0.350	0.350	0.349	0.350	0.350	***0.002***	***0.003***	***0.002***		**0.996**
19 CpGV-I07	0.349	0.349	0.349	0.349	0.349	0.350	0.350	0.349	0.350	0.350	0.349	0.349	0.349	0.349	***0.002***	***0.003***	***0.003***	***0.003***	

Baculovirus Species Demarcation and Evolutionary Speed

The phylogenetic analysis (Figure 6) demonstrated that the distances between the geographic PhopGV isolates were close to zero. This result is also reflected by the Kimura 2-Parameter (K-2-P) values of the conserved genes *polh(gran)*/*lef-8*/*lef-9* (Table 4), which are robust genetic markers for species demarcation within the family *Baculoviridae* (Wennmann et al., 2018). The K-2-P distances among PhopGV isolates ranged from 0 - 0.001. Compared to five CpGV isolates (CpGV-M, CpGV-I12, CpGV-S, CpGV-E2 and CpGV-I07), which K-2-P values ranged between 0.001 - 0.003, it can be concluded that sequence divergence among PhopGV isolates is much lower than for CpGV, though more PhopGV isolates were considered in this study. The K-2-P values between PhopGV and CpGV ranged between 0.349 - 0.351, which is typical for two different baculovirus species (Jehle et al., 2006).

Discussion

PhopGV is a baculovirus with a high number of natural hosts. All known hosts, namely *P. operculella*, *T. solanivora*, *S. tangolias*, *T. absoluta*, *E. quinoae* and *P. detectendum,* are Gelechiid species (Carpio et al., 2013; Povolny, 1967). The origin of this virus may be linked to the origin of its hosts which are native to Western South America (Graf, 1917). Although it is assumed that the virus spread worldwide together with the distribution of the hosts (Espinel-Correal et al., 2010; Zeddam et al., 1999; Kroschel and Koch, 1996; Hunter et al., 1975), REN analyses of viral DNA of geographical isolates of PhopGV revealed only low differences (Vickers et al., 1991) supporting the hypothesis that the high number of different hosts and the spatial separation of host populations over different geographic regions did not increase the genetic variability of the virus (Zeddam et al., 1999). By including a large number of PhopGV isolates and performing full genome sequencing analyses this finding could be confirmed to a very deep molecular extent. All isolates were propagated in *P. operculella* larvae before sequencing but the analysed samples CR3.1 and CR5.1 were originating from soil samples in a region where both *P. operculella* and *T. solanivora* occurred, the soil samples were propagated on *T. solanivora* (Gómez-Bonilla et al., 2011) before the last passage in *P. operculella*. Isolate IT1.1 was originating from a soil sample from an Italian tomato field and passaged in *T. absoluta*. Isolates GR1.1/GR1.2 and GR2.1 originated from soil samples from a Greek potato field and were also passaged in *T. absoluta*. Isolate YM.1 originated from infected *P. operculella* larvae from Yemen.

The genome sizes of the different PhopGV isolates were highly stable and ranged from 118,355 bp (PhopGV-CR3.1) to 119,177 bp (PhopGV-IT1.1) with a consistent number of 130 ORFs identified (Table 1).

By applying Illumina NGS, this present study sets the scale of analysing the genetically differences between PhopGV isolates to a new level of complexity and precision. The analyses of the whole genome data revealed differences for every isolate which cannot be identified by any other method previously used. The number of total SNP positions was 503 and those unique to certain isolates ranged from 6 to 34 (Table 2). These are comparably few numbers of genetic variations considering the size of the whole genome of 119 kbp. When considering SNPs, the frequency of their occurrence is a fundamental information in understanding the genetic diversity of this virus. The SNP frequencies ranged from < 0.2 to 1. For many SNP positions the reference was also supported, in other words SNP and non-SNP were supported at the same time. This finding is a hint that acquired

polymorphisms often establish without a total replacement of its prior genetic information. Genetic variation of naturally occurring baculovirus isolates was reported before, mainly by analysing sub-molar bands of REN digests for NPVs and GVs (Rezapanah et al., 2008; Simón et al., 2004; Léry et al., 1998; Smith and Crook, 1988; Lee and Miller, 1978;). The presence of genotype mixtures for the analyzed PhopGV isolates was not explicitly caused by the accumulation of PhopGV-R after propagation in Phop-IT. Isolates PhopGV-LS3 and PhopGV-CR5 could be identified as mixtures before propagation by a PCR with *egt* specific primers (data not shown). Both isolates additionally mixed with the internal virus PhopGV-R after propagation in Phop-IT. The improved technology of genome sequencing allowed an increasing detailed analysis of gene and genome variations of single baculoviruses (Gueli Alletti et al., 2017; Wennmann et al., 2017; Wennmann and Jehle, 2014; Watson et al., 2013; Eberle et al., 2009; Erlandson, 2009).

The frequency of polymorphisms could be influenced by the host in which the virus is propagated, because during virus replication single genotypes become preferred or selected over others, resulting in a mixture or a genetic drift in different SNP positions (Ebert and Hamilton, 1996). Further an activation of a host internal virus can influence the frequency of polymorphisms, like observed in the present study. Establishing of new polymorphisms may be difficult under natural selection conditions because multiple baculovirus genotypes can coexist in the same host (Erlandson, 2009; Simón et al., 2005; López-Ferber et al., 2003). Non-coding intergenic regions are liable to accumulate mutations more easily than protein coding regions which are known to be more conserved because of the evolutionary pressure to keep protein function (Chen et al., 2002). Whereas mutations in non-coding regions can have a gene regulatory effect, mutations in coding regions can have direct effects on the protein function. The Indels found inside the ORFs of PhopGV were found to be more conserved and stable in size along one group than Indels of the non-coding areas of the genome.

By analysing the SNP distribution in a given isolate (Figure 2) it is intriguing that SNP positions form patterns allowing a grouping of isolates. It revealed that some isolates share a decent number of 8 to 42 SNPs, which allowed forming four different genome groups based on SNP positions (Table 2 and Figure 3). SNPs specific for one group are not present for any other isolate of another group. This grouping system is also consistent for the presence of Indel mutations (Figure 5) and the phylogenetic tree of the isolates (Figure 6). Hence, the presence and distribution of SNPs and Indels can be used to extend the previous grouping of PhopGV isolates that was mainly based on different *egt* types (Jukes et al., 2016; Jukes et al., 2014; Carpio et al., 2013; Zeddam et al., 2013; Espinel-Correal et al., 2010). It appeared that isolate CR5.1 was most closely related to the reference PhopGV-1346, which was originally isolated from Tunisia. This finding is confirmed by the lowest number of differing SNPs and Indels between both isolates as well as the phylogenetic analysis (Figure 2, Table 2 and Figure 6).

The isolates recently collected in Italy and Greece (GR1.1, GR2.1 and IT1.1) clustered all in group 3. Nevertheless, even these isolates collected from the same geographic region show some differences: isolate GR2.1 had twelve isolate-unique SNPs, whereas isolate IT1.1 had only one isolate-unique SNP. If only the members of group 3 were compared, GR2.1 showed a number of 36 additional SNPs not occurring in the other isolates of group 3, whereas IT1.1 showed nine SNPs not present in other group 3 isolates. This finding indicates that PhopGV isolates did not need to be separated over large geographic distances to develop differences on the genetically level. But the range of polymorphisms is narrow, no matter if isolates were collected from the same area or from distant locations (Kroschel and Huber, 1996; Vickers and Cory, 1991). It appears that the different isolates collected from all over the world (Sri Lanka, Australia, South Africa, New Zealand, India, Crimea, Peru, Yemen, Kenya,

Tunisia, Venezuela, Egypt, Bolivia, Turkey, Indonesia, Peru, Costa-Rica, Canary Islands, Italy and Greece) kept a high genome stability under very different ecological conditions (Zeddam et al., 1999).

Despite the low relative frequency of polymorphisms found in the PhopGV genomes, some regional characteristics appear for group 3 isolates, which all originated from Greece or Italy (Figure 4). Only the members of group 3 (GR1.1, GR1.2, GR2.1 and IT1.1) had an insertion of 150 bp at the non-coding region between ORF 109 and 110. In addition, three Indels inside the coding region ORF 18 (*hp*), ORF 33 (*odv-e66*) and ORF 129 (*egt*) (Figure 5) as well as 32 SNPs were specific for this group (Table 2, Figure 2).

For the remaining isolates of genome group 1, 2 and 4 the internal virus genotype was present in minority and was therefore not considered when the consensus sequence based on the majority of reads was determined. Thus, except for CR3.1 and LS3.1, the subsequent analyses of Indels and the phylogenetic reconstruction was not impaired by the minor presence of PhopGV-R in the virus preparations. On the other hand, sequence comparisons of the passages of the same virus, *i.e.* LS1.1 and LS1.2 as well as GR1.1 and GR1.2 revealed, that the genomes of both passages are highly stable and acquired only very minor changes, despite propagation in the presence of an internal virus in the rearing (Table 3). This observation provides molecular evidence that *in vivo* production of PhopGV used as biocontrol agents should result in very similar virus batches with high isolate identity (Supplementary Table III 4). It further indicates that the nucleotide frequency within a certain SNP is stably inherited and likely based on selection constraints.

In conclusion for the propagated isolates which belonged to the genome groups 1, 2 and 4 no correlation between the location of collection and genetic characteristics can be predicted. In contrast, the regional characteristics present in the genome of the analysed isolates can be considered for the members of genome group 3 (GR1.1, GR1.2, GR2.1 and IT1.1) only, as they did not go into mixture with the internal virus after propagation (Larem et al., 2018).

A grouping of PhopGV isolates based on the variability of only a single gene, namely *ecdysteroid UDP–glucosyltransferase (egt)* was established previously (Jukes et al., 2016; Jukes et al., 2014; Carpio et al., 2013; Zeddam et al., 2013; Espinel-Correal et al., 2010). The *egt* typing is a reliable and consistent grouping model, which was also valid for the twelve PhopGV isolates sequenced in this study. One disadvantage of this way of grouping is that many isolates with additional polymorphisms along the genome become assigned to the same *egt* type. For example, members of the *egt* type II differed in nine other polymorphisms in the ORFs 24, 28, 32, 33, 43, 46, 94, 104 and 128. The sequenced isolates of the *egt* type II can be divided into two subgroups sharing four Indels and eight SNPs (group 1) and five Indels and 42 SNPs (group 2), respectively. Strikingly, the grouping of four isolates belonging to *egt* type III and the isolate of *egt* type I is reflected by SNP and Indel based genome groups 3 and 4, respectively. The previously identified *egt* types IV and V (Espinel-Correal et al., 2010) were not present among the tested set of isolates.

With the *superoxide dismutase* gene (*sod*) a second highly variable gene was identified in this study. Four different types of *sod* could be identified, differing in putative ORF length from 78, 213, 372 to 390 bp. Compared to other related betabaculoviruses, the predicted SOD of PhopGV is rather short: Adoxophyes orana granulovirus (NC_005038) 459 bp, Erinnyis ello granulovirus (KX859084) 474 bp and Cryptophlebia leucotreta granulovirus (MF974563) 456 bp. But different sizes were also present *e.g.* for Cydia pomonella granulovirus isolate S (KM217573) 474 bp and isolate M (KM217575) 399 bp. The specific function of this gene during the baculovirus infection is still unclear. In AcMNPV the homologue *sod* has been deleted without any apparent effect on viral replication either in cell culture

or in insects (Tomalski et al., 1991). Experiments with a deletion of the *sod* of Bombyx mori nucleopolyhedrovirus (BmNPV) resulted in a significantly delayed production of budded viruses and occlusion bodies. This finding indicated that BmNPV *sod* plays a role in efficient virus propagation in *B. mori* larvae (Katsuma, 2015). However, since *sod* is present in almost all lepidopteran baculovirus genomes a functional role for baculoviruses is very likely (Rohrmann, 2011). There is a possibility that *sod* triggers protection of occluded virus from superoxide radicals generated by exposure to sunlight in the environment, thus *sod* could act as part of a protection against DNA damage after UV-light exposure (Miller, 1997). Nevertheless, *sod* can be used as a genetic marker for PhopGV isolates discrimination and quantification.

In conclusion, PhopGV isolates collected from different climatic zones of four different continents showed highly homogeneous genomes and no differences in the number of ORFs. The application of Illumina NGS provided deep insights to a large number of different PhopGV isolates with highly conserved genomes. Based on large sample size and a grand output quality with high nucleotide coverage differences for each of the twelve sequenced isolates to the single isolate level were identified. This approach allowed establishing a new grouping system for PhopGV isolates, which is based on SNPs and Indel distribution. Learning more about the diversity and evolution of isolates of single baculovirus species will contribute to the application of baculoviruses in biological insect control as well as to their registration as biocontrol agents.

Chapter IV

Effects of a Covert Infection with an Internal Phthorimaea operculella granulovirus in Insect Populations of *Phthorimaea operculella*

Abstract

Virus infections of insects can easily stay undetected, neither showing typical signs of a disease and nor being lethal. Such a stable and most of the time covert infection with Phthorimaea operculella granulovirus (PhopGV) was detected in a *Phthorimaea operculella* laboratory colony, which originated from Italy (Phop-IT). This internal virus (named PhopGV-R) was isolated, purified and characterized on the genetic level by full genome sequencing. Further, the insect colony Phop-IT was used to study crowding effect, double infection with other PhopGV isolates (CR3 and GR1), superinfection exclusion and pupation rate under a low and high level of exposure to PhopGV occlusion bodies applied to neonate larvae. An infection with a second homologous virus (PhopGV-CR3) activated the internal virus, while a co-infection with another virus isolate (PhopGV-GR1) led to its suppression. Strikingly, Phop-IT larvae were susceptible to application of purified occlusion bodies of the internal PhopGV-R and showed a virus concentration-dependent reduction of the pupation rate. A stable covert virus infection was also detected in two additional *P. operculella* insect populations originating from Tunisia (Phop-TN) and Egypt (Phop-EG). This study shows that stable virus infections can be common for insect populations and have an impact on population dynamics because they can suppress or enable superinfection with another virus isolate of the same species.

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Introduction

Virus transmission can follow two different mechanisms. An overt virus outbreak can kill a large number of individuals of a population and may function as infective sources for other host insects, either from the same or another generation (horizontal transmission); or a virus infection does not result in the death of the infected individual and can be transmitted from the infected parental generation to their offspring (vertical transmission) (Andrealis, 1987). Horizontal virus transmission conveyed by *per os* infection initiating an acute infection cycle is well characterized for many insect viruses, including polydnaviruses, rhabdoviruses (*e.g.* sigma viruses), picornaviruses (*e.g. Dicistroviridae*), nudiviruses and baculoviruses (Valles et al., 2017; Cory, 2015; Rohrmann, 2011; Burand, 1998). But the understanding of the mechanisms involved in vertical virus transmission, is still in its infancy (Kukan, 1999). In contrast to the acute infection after horizontal transmission, virus infections exploiting vertical transmission are often covert and chronic. Such covert infections can appear either as persistent infection resulting in a continuous low level of virus replication after a primary infection, or as a latent infection when the primary infection is followed by a low level reactivation and recurrent infection (Mocarski and Grakoui, 2008). The verification of covered infections is complicated because the point of time of the analysis is crucial and can be misleading.

Covert infections have been an intriguing field of research for viruses of the family *Baculoviridae*. Baculoviruses are insect-specific dsDNA viruses mainly associated with larval stages of insects from the orders Lepidoptera (genera *Alphabaculovirus* and *Betabaculovirus*), Hymenoptera (genus *Gammabaculovirus*) and Diptera (genus *Deltabaculovirus*) (Herniou et al., 2003). Whereas overt infections result in heavily diseased larvae which succumb to baculovirus infection, covert infections can be survived and become stably established in an insect population (Sait et al., 1994), characterized by the absence of visible signs of infection (Williams et al., 2017). Covert baculovirus infections can be found in many different insect populations in the laboratory and in the field (Murillo et al., 2011; Erlandson, 2009; Burden et al., 2003; Fuxa et al., 1999; Kukan, 1999; Hughes et al., 1997). One method to obtain new baculoviruses is to collect insects from field populations and rear them in the laboratory until a virus outbreak occurs. This method has been successfully applied to isolate betabaculoviruses from various insects and environments, such as South-African strains of Phthorimaea operculella granulovirus (PhopGV-SA), Plutella xylostella granulovirus (PlxyGV-SA) and Cryptophlebia leucotreta granulovirus (CrleGV-SA) (Jukes et al., 2016; Jukes et al., 2014; Opoku-Debrah et al., 2013). The latter was obtained after overcrowding larvae of a reared *Thaumatotibia leucotreta* colony. There are also many examples for alphabaculovirus outbreaks in insect colonies which lead to successful isolations of new virus samples, *e.g.* Mamestra brassicae multiple nucleopolyhedrovirus (MbMNPV), Trichoplusia ni single nucleopolyhedrovirus (TnSNPV) and Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) (Murillo et al., 2011; Burden et al., 2003; Fuxa et al., 2002; Hughes et al., 1993).

Baculoviruses can regulate insect populations (Eldered et al., 2013; Cory and Myers, 2003; Anderson and May, 1980) and therefore it is of eminent interest to consider the natural occurring viruses present in dynamic insect populations. Overt infections are rare events in insect field populations and often correlate with mass outbreaks of the host insects (Myers, 1988; Tanada and Fuxa, 1987). Horizontal virus transmission is an effective strategy for host species with high population densities; but for low host densities such a transmission strategy would bear the risk for a given virus of becoming extinct (Anderson and May, 1981). Vertical transmission is a long-term strategy for persistence in host populations (Burden et al., 2003) and is linked to low virulence (Williams et al., 2017). But these low virulent covert infections can be triggered by stressors like temperature, humidity, nutrition, overcrowding or a secondary infection with a homologous or heterologous virus (Vilaplana et al.,

2010; Burden et al., 2003; Cooper et al., 2003; Fuxa et al., 1999; Hughes et al., 1993; Steinhaus, 1958). Evolutionary success of baculoviruses is closely related to a mixed-mode strategy involving both horizontal and vertical transmission that is widely common across a broad range of viruses, parasites, symbionts, and microbiota (Ebert, 2013).

Phthorimaea operculella granulovirus (PhopGV) [genus *Betabaculovirus*, family *Baculoviridae*] is a dsDNA virus with a circular genome of about 119 kbp, encoding 130 open reading frames (ORF) (Jukes et al., 2016; Croizier et al., 2002). It can infect several members of the lepidopteran family Gelechiidae like *Phthorimaea operculella*, *Tuta absoluta*, *Tecia solanivora* and others (Carpio et al., 2013; Lacey and Kroschel, 2009; Povolny, 1967). Recently, a comprehensive comparison of 14 genome sequences of different PhopGV has been published, resulting in the identification of four phylogenetic lineages which also corresponded to the distribution and prevalence of single nucleotide polymorphisms and insertion and deletion mutations (Larem *et al.*, 2018). The virus-encoded gene *ecdysteroid UDP-glucosyltransferase* (*egt.* ORF129) is of particular interest because its gene expression leads to the inactivation of ecdysteroid, which acts as a molting hormone in host development (Burand and Park, 1992; O'Reilly and Miller, 1989). In the PhopGV genome the *egt* gene can occur in five different size variants, whith ORF lengths between 861 and 1,353 bp (Juke *et al.*, 2014). This variability of the *egt* gene has been used also to group and differentiate PhopGV isolates according to these five *egt* types (I-V) (Jukes et al., 2014; Carpio et al., 2013). Another highly variable gene is *superoxide dismutase* (*sod*, ORF54).

In this study a new PhopGV isolated from a persistent covert infected laboratory rearing of *P. operculella* (Phop-IT) was characterized. This infection study was covert in most cases, which means that no virus-dead individuals were detected but a potential for an active overt virus outbreak was present. In addition, the infection was persistent over many generations. Transmission of virus from parents to the next following generation was most likely carried out over eggs where a transovarial and transovum pathway is possible. Transovarial means a transmission from maternal parent to progeny embryos within the eggs, whereas transovum involves contamination of the exterior egg surface with viral particles that infect neonate larvae as they ingest the chorion during hatching (eclosion) (Williams et al., 2017; Cory and Myers, 2003; Kukan, 1999).

Further the effects of this virus to the insect population in terms of development and susceptibility for secondary virus infections were studied. It was demonstrated that a mixed mode of interaction is possible and the internal virus either can be activated by a secondary virus and becomes overt as a mix of both viruses or can be suppressed by other virus strains within the same virus species. Overt infection with the internal virus lowered the pupation rate of *P. operculella* significantly. At high virus dosage the internal virus, a second virus and a mixture of both viruses all prevented pupation to a rate close to zero percent. Activation and/or suppression of internal viruses as found with PhopGV are crucial for the understanding of virus population dynamics and viral ecology. In addition, they argue for a careful quality management when baculoviruses are produced as biological control agents under *in vivo* conditions.

Material and Methods

Insects

A rearing of the potato tuber moth *Phthorimaea operculella* was established at the JKI in 2014. The colony was obtained from COOP. TERREMERSE, Bagnacavallo (Italy) and was based on insects collected in the Emilia Romagna, Ravenna. This strain was named Phop-IT. The larvae were kept on potato slices at 26 °C and 16/8 h light/dark conditions until pupation. Potato slices were placed on sand to allow pupation outside of the potato and to ease the collection of the pupa with a mesh. After hatching, the adults were transferred into cylinders (⌀ 24.5 cm, height = 18 cm), covered with a dark plastic bag, and placed on a tray. A piece of cotton wool, watered with 10% sucrose solution was provided to feed adults. The top end of the cylinder was closed with fine gauze, which was permeable for egg laying on a filter paper placed on top of the gauze outside of the cylinder. This technique allowed an exchange of the egg paper with a fresh one without opening of the cylinder. After the collection of the egg paper, the eggs were incubated at either 20 °C or 26 °C which allowed manipulating the hatching day of the neonate larvae. Sterile material was used for the rearing in order to avoid a contamination. To eliminate the risk of contamination across generations, single use material was applied.

In addition to the strain Phop-IT, two additional *P. operculella* laboratory rearings were established from insects collected from a potato storage room in Tunisia (Phop-TN) and field collections from Egypt (Phop-EG). The different insect populations were kept separately but under the same rearing, temperature and light conditions. To avoid an interaction between the different insect strains, the three rearings were maintained not only spatially separated from each other but were also handled by different persons.

Virus

Different isolates of the Phthorimaea operculella granulovirus were used in this study: (i) PhopGV-R (covert virus of the Phop-IT rearing), (ii) PhopGV-CR3.1 (origin Costa Rica; Gómez-Bonilla et al., 2011) and (iii) PhopGV-GR1.1 (field collection; Greece). PhopGV-CR3.1 and PhopGV-GR1.1 were the first larval passages of OB samples of isolates PhopGV-CR3 (Public University of Navarra, Agrobiotechnology Institute, Pamplona, Spain) and PhopGV-GR1 (Hellafarm SA, Attica, Greece), respectively. Larval passages were conducted with the laboratory colony Phop-IT. The propagated PhopGV isolates PhopGV-CR3 and PhopGV-GR1 received the suffix ".1" to indicate their passage through the host PhopIT during virus propagation.

Co-Propagation of PhopGV-CR3 and PhopGV-GR1

The surface of each potato disc (4.3 cm Ø, 0.5 cm thickness) was inoculated with 200 µl OB suspension (1×10^4 OB/ml) of either CR3 or GR1. Twenty neonate larvae of *P. operculella* were transferred onto one potato disc using a fine paint brush. The potato discs were kept at 26 °C, 60% RH and 16/8 h light/dark photoperiod. After six days the inoculated larvae showed typical baculovirus infection symptoms, such as loss of mobility, decreased feeding rate and change in colour from green to bright white followed by sluggishness and flaccidity (Gómes Valderrama et al., 2017; Lacey et al., 2011; Briese 1981; Reed, 1969). From this point the potato discs were examined daily and dead

larvae were collected. The experiment was repeated until a total of 80 virus-killed larvae of each virus treatment was obtained.

Virus Purification

For the homogenization of the pooled larval material an electric homogenizer (IKA T25 digital Ultra Turrax) was used. The purification of the viral occlusion bodies (OB) followed a modified protocol of Smith and Crook (1988). The obtained homogenate was treated with sodium dodecyl sulphate (SDS) (10% w/v) to a final concentration of 0.5% and kept on ice for 30 min. After 3 min incubation in an ultrasonic water bath, the sample was filtered through a sandwich filter made of cotton and cotton wool. To minimize the loss of viral OBs the filter was washed with additional volumes of 0.5% SDS or dH_2O. After several washing steps including centrifugation at 22,000 g for 15 min and resuspension of the pellet with dH_2O, larval debris was removed from the OB sample. The obtained OB suspension was loaded onto a glycerol step gradient: 50%, 55%, 60%, 70%, and 80% (v/v) top to bottom. Prepared step gradients were loaded into an A-4-62 swing-out rotor and centrifuged at 3,200 × g for 45 min in an Eppendorf 5810R centrifuge. OBs were visible as a light grey band between 55% and 60% glycerol concentration. This band was collected and washed by additional centrifugation steps to remove the glycerol from the sample.

DNA Isolation from Occlusion Body

Separately for each isolate PhopGV-R, PhopGV-CR3.1 and PhopGV-GR1.1, 150 µl of purified OB suspension (1.6×10^{12} OB/ml) derived from infected Phop-IT larvae were used for DNA isolation, according to the protocol of Jehle and Arends (2002).

Whole DNA Isolation from *P. operculella* Eggs, Larvae, Pupae and Adults

DNA isolation from various sized batches of eggs (n = 5, 10, 15, 20 and 30), individual larvae (n = 150), individual pupae (n = 50) and individual adults (n = 50) were performed by following a slightly modified protocol of Ron's tissue DNA Mini Kit (Bioron GmbH, Ludwigshafen, Germany). First, samples were grinded thoroughly under presence of the kits lysis buffer using a pistil. Then, the obtained homogenates were centrifuged for 2 min at maximum speed (20,800 × g) to remove any larval debris. Supernatants were used for whole DNA purification following the instructions of the kit.

Complete *egt* Gene Amplification

The oligonucleotide primer pair egtF (5'-GAG TCG AGC CAA TTT TGT TTG CG-3') and egtR (5'-GCA ACG ATG ATC TCA TAT ATG AGC-3') (Jukes et al., 2014), flanking 204 bp up- and downstream ORF 129, were used for PCR amplification of the *ecdysteroid UDP-glycosyltransferase* (*egt*) gene region. The reactions comprised 10 µl of 5 x Green Buffer (including $MgCl_2$ 7.5 mM), 1 µl dNTPs (10 mM each), 1 µl of forward and reverse primer (10 µM), respectively, 50 ng DNA template and 0.25 µl Go Taq Polymerase (5 U) (Promega GmbH, Mannheim, Germany). Reactions were filled up to a final volume of 50 µl using ddH_2O. PCR reactions were initiated by a denaturation step at 94

°C for 4 min, followed by 33 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min and a final extension step at 72 °C for 5 min. Amplified products were visualised by agarose gel (1%) electrophoresis at 90 V for 45 min in 1x TAE buffer (40 mM Tris-acetate, 20 mM acetic acid, 1mM EDTA) stained with Midori Green Advance (Nippon Genetics Europe GmbH, Düren, Germany). Visualisation and caption of the gel images was done with ChemoCam Imager ECL UV trans-illuminator and software (INTAS Science Imaging Instruments GmbH, Göttingen, Germany).

DNA Restriction Endonuclease Digests

The PCR amplification products were purified using the Clean & Concentrator Kit-25 (Zymo Research Europe GmbH, Freiburg, Germany). DNA concentration and quality was determined by measuring 1 µl of the DNA samples in a Nanodrop 2000c Spectrometer (Thermo Scientific, Wilmington, Delaware USA). DNA restriction endonuclease (REN) digests comprised 17.5 µl DNA in dH_2O (800 ng), 2 µl reaction buffer, 0.2 µl of 10 µg/µl BSA and 0.5 µl *Alu*I (Promega GmbH, Mannheim, Germany). The reaction was incubated for 5.5 h at 37 °C and loaded directly on a 1% agarose (TAE) gel.

Mixed Infection of Phop-IT Neonate Larvae

For setting up infection experiments, 200 µl of a PhopGV OB suspension (1.3×10^4 OB/ml) were applied to inoculate the surface of a potato disc (⌀ 43 mm, 5 mm thickness). Either PhopGV-R or an equal mixture of 100 µl PhopGV-R and 100 µl PhopGV-GR1.1 was applied as inoculum. By using a knife, several parallel cuts were applied to the surface of the potato slices in order to facilitate the finding of the test larvae at 7 days post infection (dpi) and 14 dpi. Twenty neonate larvae were placed on each potato slice using a fine brush. Each of the potato slices was kept in a Petri dish and incubated at 26 °C and 16/8 h light/dark photoperiod. This experiment was performed with three independent repetitions for each virus treatment and both evaluation time points. After 7 and 14 days larvae were collected from each treatment and conducted to total DNA isolation as mentioned previously. DNA samples were used for the identification of *egt* type I to V by partial PCR amplification.

Crowding Experiments with Phop-IT

Different numbers (5, 10, 15, 20, 30 or 40) of neonate larvae of the *P. operculella* population Phop-IT were placed on a potato disc (⌀ 43 mm and 5 mm thickness). Each of the potato slices was kept in a Petri dish and incubated at 28 °C and 16/8 h light/dark photoperiod. The number of healthy larvae, virus-infected larvae and pupae was recorded after 13 days. For each tested number of neonate larvae, eight potato slices were provided.

Whole Genome Sequencing of Isolates

About 150 ng/sample of genomic DNA of PhopGV-R, -CR3.1 and -GR1.1 were sent for whole genome sequencing (StarSEQ GmbH, Mainz, Germany) with 150 bp read length and paired-end

options (Larem et al., 2018). Library preparation and bioinformatic analysis of sequencing data followed the protocol as described by Larem et al. (2018).

Results

Identification of a Covert Infection in Phop-IT

After 48 weeks (12 generations) of maintaining Phop-IT in the laboratory, 80 larvae were collected directly from the rearing during a time period of 20 weeks (4 generations). All selected larvae showed phenotypical signs of baculovirus infection like lethargic behaviour, whitish colour and tumid appearance. Sampling for PhopGV detection was done by collecting single larvae of the developmental stages L3/L4, followed by DNA purification and a PCR with specific primers for PhopGV *egt* gene fragment. The PCR results confirmed the phenotypical observation of a virus infection of the collected *P. operculella* larvae. The size of the amplified *egt* fragment was 1,557 bp, which included a 204 bp flanking region to the *egt* ORF. Thus, the *egt* coding region was predicted to be 1,353 bp, indicating an *egt* type II of the isolated PhopGV according to Carpio et al. (2013). This finding was corroborated by digesting the PCR product with *Alu*I, resulting in three *Alu*I fragments of 413 bp, 462 bp and 682 bp, as it is typical for the *egt* type II (Figure 1). This internal virus in the rearing of Phop-IT was termed PhopGV-R. All collected PhopGV positive samples generated the same DNA restriction pattern (data not schown).

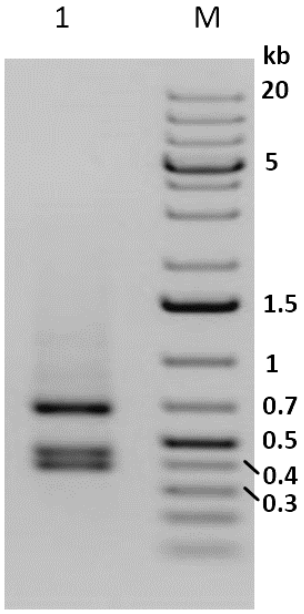

Figure 1. Agarose gel (1%) electrophoresis of the *Alu*I digest of the *egt* PCR product from purified DNA of collected single larvae from the insect population Phop-IT (lane 1). Size marker (lane M) GeneRuler 1 kb plus DNA ladder (Thermo Scientific). The gel image was inverted for better visualization of the PCR fragments.

Sequence Analysis of PhopGV-R

The collection of 80 larvae was pooled and homogenized before the virus purification method. This internal virus in the rearing of Phop-IT was termed PhopGV-R. In the following the internal virus PhopGV-R was isolated and purified DNA was subjected to Illumina sequencing. The GC content of the sequenced isolate PhopGV-R was 35.7% and showed no difference compared to other PhopGV isolates. General information about the sequenced PhopGV-R is given in Table 1. With a genome length of 119,080 bp, encoding for 130 ORFs (Supplementary Table S IV 1), PhopGV-R was close to the genome length 119,061 bp of PhopGV-GR1.1 and belonged to one of the longer PhopGV sequences ranging between 118,355 bp (PhopGV-CR3.1) and 119,177 bp (PhopGV-IT1.1), which was the range of the genome length of sequenced PhopGV isolates till now (Larem et al., 2018). Sequence analyses confirmed the *egt* (ORF 129) length of 1,353 bp (type II) of PhopGV-R. The two other PhopGV isolates used in this study, PhopGV-GR1.1 and PhopGV-GR1.1, showed *egt* ORFs of 1,053 bp (type III) and a mixture of both types, respectively (Larem et al., 2018; Jukes et al., 2014; Carpio et al., 2013).

Table 1. General information about the sequenced PhopGV-R isolate and two additional PhopGV isolates used for this study.

Isolate (PhopGV-)	Origin	Reads Assembled	Coverage / nt	Consensus Seq. Length	Accession No.	Source/ Reference
R	Darmstadt	1,163,983	1,284	119,080	-	JKI
CR3.1	Costa Rica	936,612	1,187	118,355	-	UPNA / Gómez-Bonilla et al., 2011
GR1.1	Greece	1,647,866	2,076	119,061	-	Hellafarm

Single Nucleotide Polymorphisms (SNPs)

For the detection of SNPs only positions with a variable frequency above 5% were included in this analysis; SNPs with lower frequency were considered as minor variation and were not further treated. Compared to the published reference sequence PhopGV-1346, the analysed sample PhopGV-R showed a total number of 97 SNP positions (Supplementary Table IV 2). Eighty SNPs were located within open reading frames (ORF), of which 46 SNPs caused an amino acid change. Forty-two SNPs were located inside ORFs with known function, of which 21 SNPs resulted in amino acid changes of 14 different ORFs (Table 2). Nine out of these 14 ORFs (*p49, p74, lef-1, helicase-1, lef-4, vp39, vlf-1, DNApol and lef-8*) belonged to the core gene family, which includes ORFs shared by all baculoviruses (Javed, 2017).

Table 2. Single nucleotide polymorphisms (SNPs) in open reading frames (ORF) with known functions of PhopGV-R in relation to the reference isolate PhopGV-1346 (NC004062). SNPs occurred in the majority of reads (> 51%) with one exception (*) where minority (22.4%) was detected. The occurrence of the respective SNPs in PhopGV-CR3.1 and PhopGV-GR1.1 is indicated with (+) for majority and (-) for minority number of reads supporting this SNP.

ORF	Encoding Gene	Direction	Position	Change (nt)	Change (aa)	Present	
						CR3.1	GR1.1
13	*p49*	reverse	9,514	A -> G	Tyr -> His	+	
24	*pe38*	reverse	16,657	T -> G	Lys -> Ans	-	
27	*efp*	forward	21,377	C -> A	Thr -> Lys	-	
41	*MP-nase*	reverse	35,905	C -> T	Asp -> Asn	+	
54	*sod*	reverse	45,394	G -> A	Leu -> Phe	-	
54	*sod*	reverse	45,586	A -> T	Cys -> Ser	+	+
55	*p74*	reverse	47,389	C -> A	Arg -> Ile	-	
66	*lef-1*	reverse	56,063	C -> T	Cys -> Tyr	-	
66	*lef-1*	reverse	56,420	A -> G	Val -> Ala	-	
82	*helicase-1*	forward	68,953	C -> G	Thr -> Arg	+	
87	*lef-4*	reverse	75,799	G -> A	Ala -> Val	-	
87	*lef-4*	reverse	76,009	C -> A	Ser -> Ile	+	+
87	*lef-4*	reverse	76,284	C -> T	Met -> Ile	+	+
87	*lef-4*	reverse	76,364	T -> C	Tyr -> Ala	-	
88	*vp39*	forward	77,124	T -> G	Ser -> Ala	+	
99	*vlf-1*	forward	85,078	T -> G	Tyr -> Asp	+	+
99	*vlf-1*	forward	85,232	A -> C	Asn -> Thr	-	
103	*DNApol*	reverse	87,530	G -> C	Gln -> Glu	+	+
105	*lef-3*	reverse	93,422	G -> C	Asn -> Lys	+	+
105	*lef-3(*)*	reverse	94,066	A -> T	Leu -> Met	-	
121	*lef-8*	reverse	111,135	C -> T	Gly -> Arg	+	

Changes in the nucleotide sequence, leading to altered amino acid sequences of the resulting proteins with known function, were found in four genes involved in the replication (*lef-1*, *helicase-1*, *DNApol* and *lef-3*), in three genes involved in transcription (*pe38*, *lef-4* and *lef-8*), in one gene for oral infectivity (*p74*), one for cell-to-cell infectivity (*efp*), in two auxiliary genes (*MP-nase* and *sod*) and in three genes involved in packing, assembly and release (*p49*, *vp39* and *vlf-1*) (Table 2) (Ferrelli, 2012).

All these SNPs, causing amino acid changes, were also found in PhopGV-CR3.1 but only eleven of them occurred in the majority number of the supporting reads, the remaining ten SNPs occurred in the minority number of reads. PhopGV-GR1.1 shared six SNPs with PhopGV-R, which were all supported by the majority number of reads (Table 2).

Insertions and Deletions (Indels)

PhopGV-R showed Indel mutations in relation to the reference isolate PhopGV-1346 (NC004062). These Indelscomprised seven deletions and two insertions allocated to seven different ORFs (24, 28, 33, 43, 94, 123 and 129) of the PhopGV genome (Figure 2). The distribution of these Indels is typical for an isolate of genome group I of PhopGV (Larem et al., 2018); but two Indels namely ORF 94 and ORF 123 were observed for PhopGV-R in addition. Four ORFs were coding for proteins of known function, namely ORF 24 (*pe-38*), a gene involved in transcription, ORF 33 (*odv-e66*), coding for a structual protein involved in oral infectivity, ORF 94 (*vp91*) involved in packing assembly and release and ORF 129 (*egt*), which is an auxiliary gene (Ferrelli et al., 2012; Russel and Rohrmann, 1997). ORF 24 (*pe-38*) showed a deletion of an Alanin amino acid (aa) residue (ΔA), ORF 33 (*odv-e66*) had two deletions (ΔTP and ΔQPQPAP), ORF 94 (*vp91*) one deletion (ΔNLDKIT). ORF 129 (*egt*) contained one insertion (+QV), resulting in an *egt* type II with a length of 434 bp (Figure 2) (Gómez-Bonilla *et al.*, 2011). Further Indels were present in three ORFs coding for proteins with unknown function. Two deletions (ΔGG and ΔAKK) in ORF 28, one deletion in ORF 43 (ΔPT) and one insertion in ORF 123 (+STL). Five Indels were also present in PhopGV-CR3.1 (ORF 24, 28, 33, 43 and 129). PhopGV-GR1.1 showed no accordance with the Indels of PhopGV-R displayed in Figure 2.

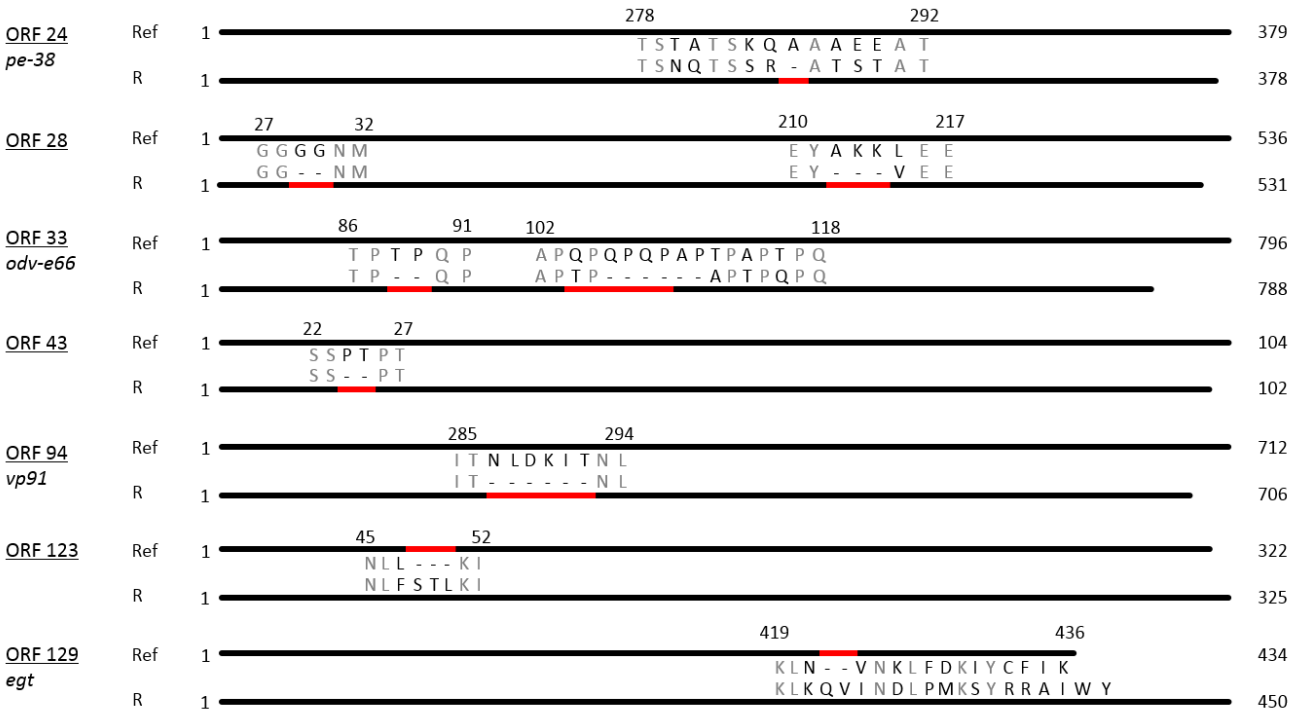

Figure 2. Diagrammatic representation of amino acid sequences representing ORFs with Indel occurrence in PhopGV-R aligned with the respective ORFs from the reference isolate NC004062. The total protein length of each ORF is shown to the right, while the numbers above each insertion/deletion region represent the corresponding amino acids from the aligned reference sequence. Deletions/insertions are indicated by a red bar.

Sod Frequency

The protein superoxide dismutase (*sod*) in general has a function of removal of radicals which produce cell damaging superoxide. It is present in almost all aerobe living organisms (Fukai and Ushio-Fukai, 2011) and also in almost all lepidopteran baculovirus genomes (Rohrmann, 2011). There is no evidence that baculovirus coded *sod* has the same function as the *sod* in other species and further that a functioning baculovirus *sod* gets expressed (Tomalski et al., 1991). Different PhopGV isolates have *sod* ORFs with varying length (Larem *et al.*, 2018). The *sod* (ORF 54) of PhopGV-R was detected in two variations: *sod* I (78 bp) and *sod* IV (390 bp) with a frequency of 0.09 and 0.91, respectively (Figure 3). The other two *sod* types occurring in PhopGV, *sod* types *sod* II (213 bp) and *sod* III (372 bp) (Larem et al., 2018), were not present for PhopGV-R.

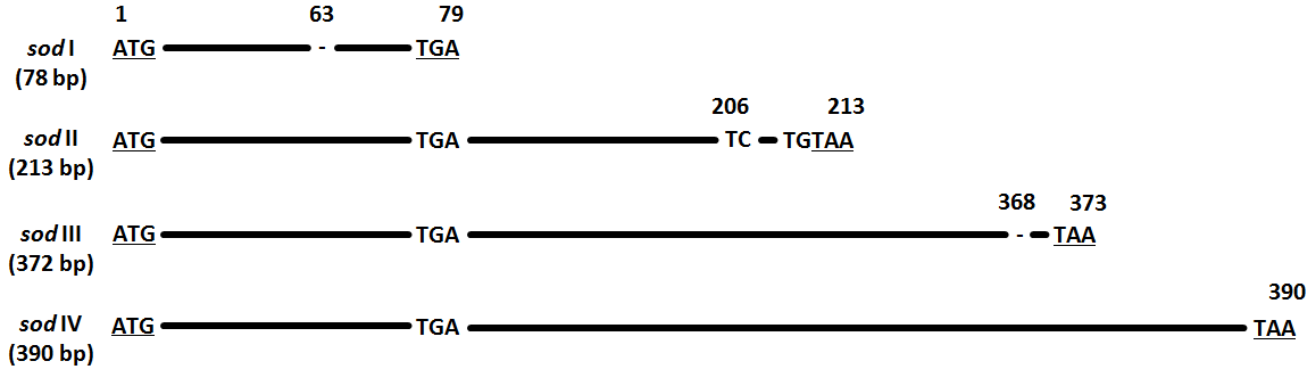

Figure 3. Nucleotide alignment of the four *sod* types (I-IV). Nucleotide positions and differences are displayed in relation to *sod* type IV (390 bp). Start and stop codons are underlined.

Virus Propagation Produced Double Infections

Propagation of isolate PhopGV-CR3 in neonate larvae of Phop-IT and purification of OB from pooled, infected larvae, resulted in the sample, PhopGV-CR3.1. Whereas PhopGV-CR3 was considered a 100% pure *egt* type III. PCR analysis of the *egt* gene fragment of the offspring virus sample PhopGV-CR3.1 revealed a mixture of *egt* type III, as typical for PhopGV-CR3, and egt type II, pointing to the presence of PhopGV-R (Figure 4). Indeed complete genome sequencing of PhopGV-CR3.1 confirmed a mixture of both viruses, when genotype quantification was based on the different number of *Alu*I cleavage sites (AG'CT) on *egt* (ORF 129). PhopGV-CR3 has one recognition site for *Alu*I, whereas PhopGV-R in contrast bears two recognition sites. The total number of reads were counted for the occurrence of this second *AluI* site to ensure a quantification of these two isolates in a mixture. The coverage of 1,203 reads supported 962 times the second *Alu*I site which is present in PhopGV-R (*egt* type II) but not in PhopGV-CR3 (*egt* type III). It was estimated that an inoculum of 100% PhopGV-CR3 resulted in 20% PhopGV-CR3 and 80% internal virus propagation (see below). The *egt*-based quantification of genotypes could be confirmed by *sod* type analysis; isolates which showed a mixture of different *egt* types showed also different *sod* types (ORF 54). Thus, a recombination of *egt* (ORF 129) only during propagation in Phop-IT is not plausible. A benefit of *Alu*I site quantification was that it could be visualized also on a gel after digestion of *egt* PCR products (Figure 4).

For this study *egt* type II with 1,353 bp in length and type III with 1,053 bp were of further interest, while other types (I, IV and V) did not occur. *Egt* type II showed three *Alu*I fragments of 413 bp, 462 bp and 682 bp in length (Figure 4, lane 3), whereas *egt* type III had two *Alu*I fragments with 575 bp and 682 (Figure 4, lane 1). A mixture of both types was generating four fragments (Figure 4, lane 2). The sum of the *Alu*I fragments were larger than the ORF length of *egt,* because the *egt* PCR products covering up- and downstream flanking 204 bp.

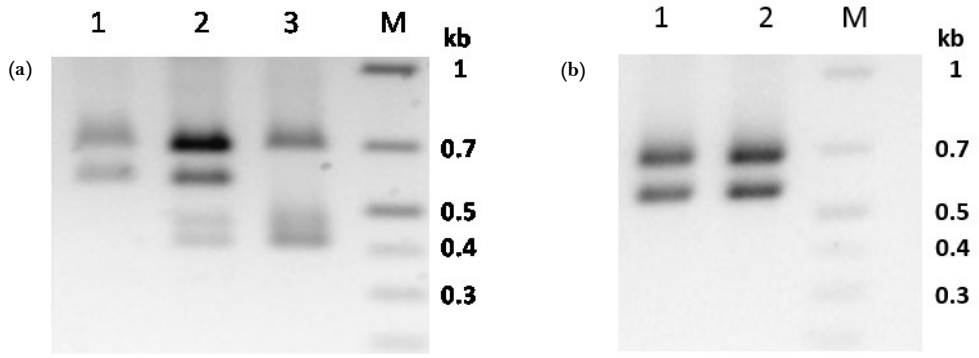

Figure 4. Agarose gel (1%) electrophoresis of the *Alu*I digested PCR product *egt* of PhopGV-CR3 (a) before propagation, *egt* type III (1) and after propagation in Phop-IT, mixture of *egt* type II and III (2), and of PhopGV-R, *egt* type II (3). *Alu*I digested PCR product *egt* of PhopGV-GR1 (b) before propagation, *egt* type III (1) and after propagation, *egt* type III (2), in Phop-IT. M = GeneRuler 1 kb plus DNA ladder (Thermo Scientific). The gel images were inverted for better visualization of the PCR fragments.

PhopGV-GR1 Suppresses Replication of PhopGV-R

Whereas infection of Phop-IT larvae with PhopGV-CR3 led to a mixed infection with the majority of PhopGV-R in the viral progeny, a differnt result was obtained when PhopGV-GR1 was propagated in Phop-IT. The resulting virus progeny PhopGV-GR1.1 was free of any contamination with internal virus. PhopGV-GR1.1 showed the same *egt* type III before and after propagation in Phop-IT larvae (Figure 4b). This effect was double checked by whole genome sequencing of the DNA obtained from a propagation batch of PhopGV-GR1.1 (see below). The virus preparation of the pooled larvae did not show any contamination with internal PhopGV-R after an infection with PhopGV-GR1. A simple recombination of *egt* (ORF 129) can be excluded because analysis of the whole genome sequencing data of PhopGV-GR1.1 showed only one *sod* type, a contamination with PhopGV-R would introduce a second *sod* type and PhopGV-GR1.1 shows an insertion of 150 nt between ORF 109 and ORF 110 which can be used for discrimination against PhopGV-R (see chapter III).
After single larvae were infected with PhopGV-GR1.1, no internal virus was present at 7 and 14 dpi (data not shown).

To confirm the observation of suppression of the internal virus PhopGV-R by PhopGV-GR1, neonate larvae of Phop-IT were infected with either the purified PhopGV-R or a mix (1:1) of PhopGV-R and PhopGV-GR1. Larvae were collected after 7 dpi and 14 dpi and a whole DNA extraction was done. The *egt-specific PCR* fragments were then digested with *Alu*I to confirm the identity of the isolated virus (Figure 5). Larvae infected with PhopGV-R showed only *egt* type II restriction patterns. Larvae infected with a mixture of PhopGV-R and PhopGV-GR1.1 showed only restriction patterns specific for *egt* type III. Neither *egt* type II alone nor mixtures of type II and III were observed.

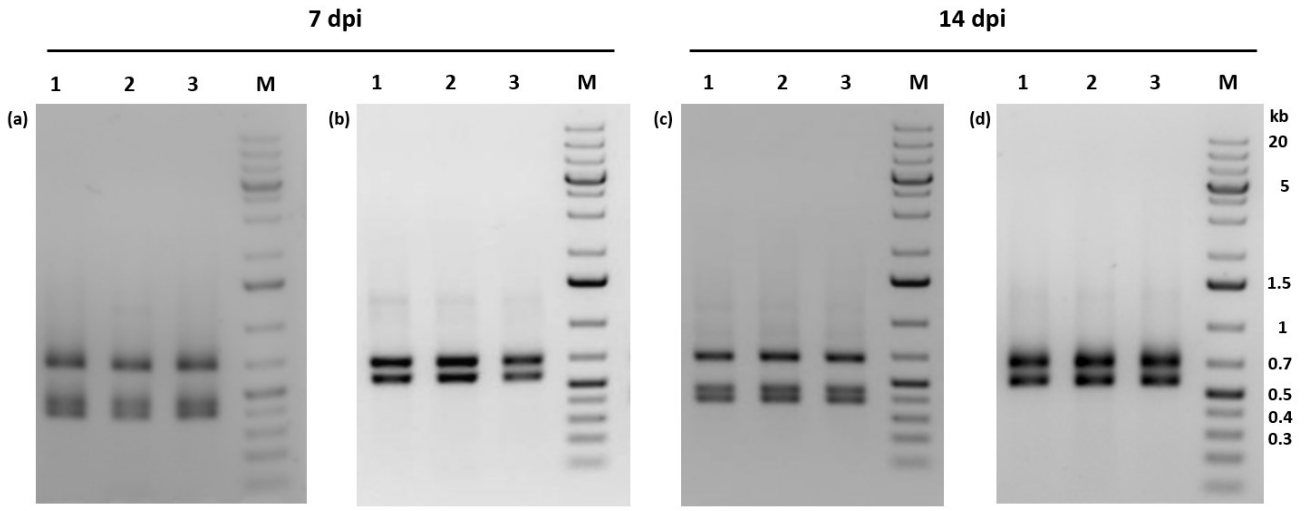

Figure 5. Agarose gel electrophoresis (1%) of *Alu*I digests of egt-specific PCR fragments obtained from infections of Phop-IT larvae using single virus and co-infections. Virus progeny was analysed after 7 and 14 dpi. Larvae were per orally infected with either PhopGV-R (a, c) or a 1:1 mixture of PhopGV-R and PhopGV-GR1 (b, d). Detection experiments were performed in triplicates (lanes 1-3), each replicate consisted of 20 infected larvae. M = GeneRuler 1 kb plus DNA ladder (Thermo Scientific). The gel images were inverted for better visualization of the PCR fragments.

Co-Propagation of PhopGV-CR3 and PhopGV-GR1

Upon propagation in Phop-IT larvae, e*gt* type III of isolate PhopGV-CR3 was replaced by *egt* type II. This effect was due to replication of the internal virus PhopGV-R, which is an *egt* type II virus (see below). After propagation, the resulting progeny PhopGV-CR3.1 considered of an additional *egt* type (II), whereas PhopGV-GR1.1 kept the same *egt* type.

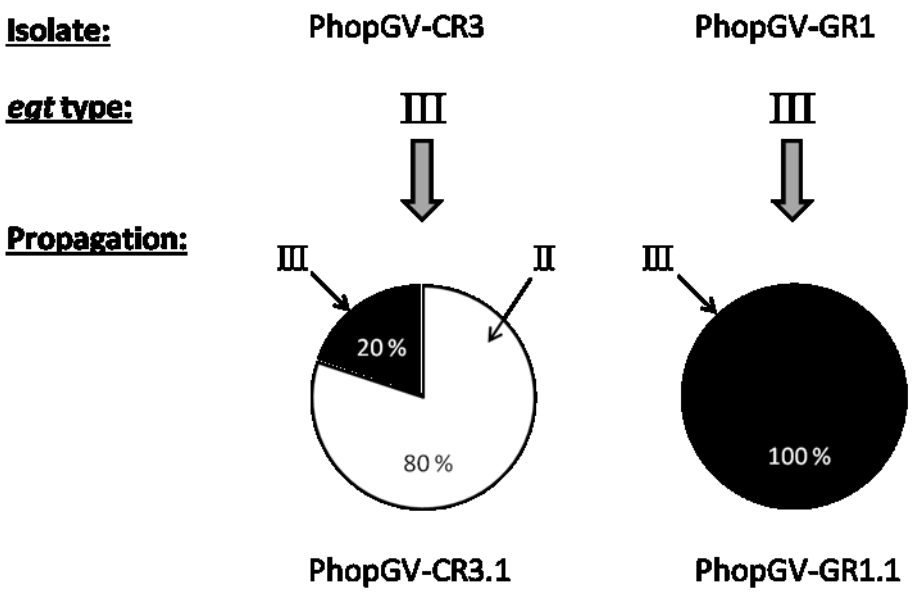

Figure 6. Quantification (%) of the *egt* types of PhopGV-CR3 and -GR1 based on the *de novo* assembled reads of the Illumina sequencing data after propagation in *P. operculella* population Phop-IT. The *egt* type is indicated with Roman numbers.

Phylogenetic Analysis

A Minimum Evolution phylogenetic reconstruction was performed with the three whole genome nucleotide sequences of PhopGV-R, PhopGV-CR3.1, and PhopGV-GR1.1 compared to the two reference isolates PhopGV-1346 (NC004062) and PhopGV_SA (KU666536). The phylogenetic tree resulted in a clustering of the PhopGV isolates following the *egt* types (I-III). PhopGV-CR3.1 and PhopGV-R grouped together because PhopGV-CR3.1 contains 80% of the R isolate. The reference isolate PhopGV-1346 belongs to *egt* type I. PhopGV-GR1.1, which belongs to *egt* type III showed the greatest evolutionary distance to all other isolates (Figure 7).

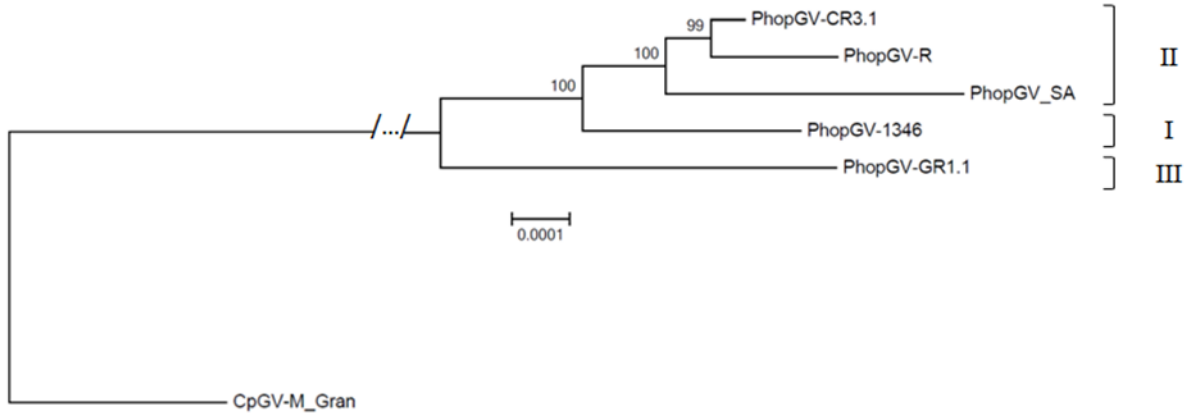

Figure. 7. Minimum evolution phylogenetic tree of different PhopGV isolates. Whole genome nucleotide information was used for conducting the minimum evolution method with Kimura 2-parameter distances and a bootstrap value of 500 replicates (Rzhetsky and Nei, 1992). The optimal tree with the sum of branch length = 0.19859 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The Neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree. All ambiguous positions were removed for each sequence pair. There was a total of 119,898 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). The tree was rooted by using the *granulin* gene of CpGV-M as an outgroup. The *egt* types of the PhopGV isolates were added with Roman numbers (I-III).

Crowding of Larvae had no Effect on Overt Virus Infections

To test whether crowding of larvae of Phop-IT had an effect on the development of overt infections, 5–40 neonate larvae were placed on a potato disc and the number of healthy larvae, infected larvae and pupae was recorded after 13 days. The rate of lethally infected larvae ranged between 1.1% and 4.1% with crowding but the differences were not signigicant (Table 3). Only the pupation rate was significantly reduced at higher densities of 40 individuals per potato disc compared to low densities of 5 to 15 larvae per disc (t-test, Bonferroni, p = 0.037). In addition, the total number of survivors was significantly reduced by 23% (t-test, Bonferroni, p = 0.029), when crowding of 40 and 5 larvae per disc were compared. No significant differences were observed for the number of healthy larvae und the number of virus infected larvae.

Table 3. Effect of crowding of Phop-IT larvae on a potato disc (⌀ 43 mm) at 28 °C after 13 days. The letters (abc) indicate statistically significant differences (t-test, Bonferroni) within the different categories. N = number of independent replicates, n = total number of tested larvae

Larvae / Disc	**5**	**10**	**15**	**20**	**30**	**40**
N	8	8	8	8	8	8
n	100	180	285	380	540	680
% Larvae infected	1.1 ±4.6	1.7 ±3.9	1.8 ±3.8	2.1 ±3.8	3.2 ±4.4	4.1 ±4.2
% Larvae healthy	9 ±16.5	4.4 ±12.5	4.6 ±5.9	2.6 ±4.5	2.8 ±3.2	7.2 ±9.8
% Pupae	67 ±29.2a	70.6 ±21a	68.1 ±15.3a	59 ±15.8ab	55.7 ±10.3ab	50.5 ±11.5bc
% Living	77 ±25.4a	76.1 ±17.9ab	73.7 ±15.8ab	63.7 ±16.2ab	61.8 ±9.9ab	59 ±11.5b

Pupation Rate

To test whether pupation rate is influenced by the virus concentration applied to neonate *P. operculella* larvae and if different virus isolates or mixtures of two isolates have a diverse effect, pupation rate was recorded for Phop-IT. Pupation rate was scored after 7, 14 and 21 days. After 7 days no pupation was observed, neither for the control nor for the virus treated group. The untreated control larvae of *P. operculella* (Phop-IT) reached a pupation rate of 38.8% after 14 days and 61.3% after day 21 in relation to the total number of individuals which were initially introduced to the experiment. No significant differences to the control group were visible for the virus-treated samples under low virus pressure (1.3×10^4 OB/ml). A significant reduction in the pupation rate was observed for the individuals treated with PhopGV-R (t test: $p = 0.0009$) and for those treated with PhopGV-GR1 (t-test: $p = 0.0273$) on day 21 (Figure 8a). The reduction of pupation rate from control to PhopGV-R treated individuals was 34.7% from 61.3% to 40% on day 21. The effect of PhopGV-GR1 was smaller with a reduction of 26.3% from 61.3% control to 45.4% in the treated group. At higher virus pressure (1.3×10^6 OB/ml) pupation rate of the control group was 31.1% after 14 days and 52% after 21 days (Figure 8b). All virus-treated individuals showed a significant reduction in the pupation rate (t test: $p < 0.001$) after 14 and 21 days, when pupation rate dropped close to zero for all treatments (Figure 8b).

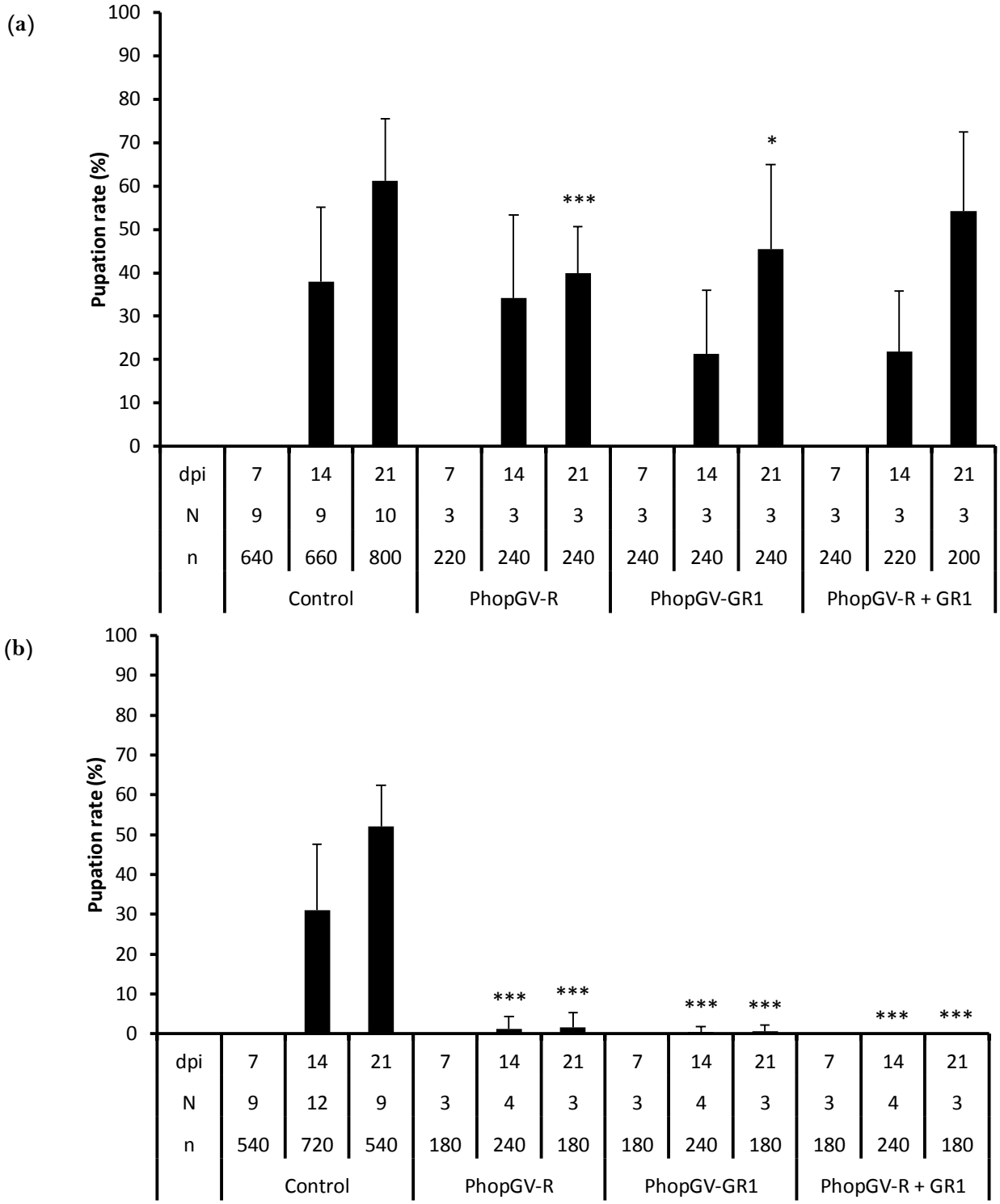

Figure 8. Pupation rate (%) of Phop-IT at three different points of time (7, 14 and 21 dpi) under exposure to PhopGV-R, PhopGV-GR1 and mixture of PhopGV-R and PhopGV-GR1 at a low concentration (1.3×10^4 OBs/ml) (a) and high concentration (1.3×10^6 OBs/ml) (b). Independent repetitions (N) and total number of tested individuals (n) are indicated. The standard deviation (SD) is indicated as well as the significance ($p < 0.05$) towards the control. Levene's test for homogeneity of variance was performed before the pairwise comparisons using t tests with pooled SD, *p*-value adjustment method: Bonferroni. Significance level (α) = 0.05 (*), 0.01 (**) and 0.001 (***).

Occurrence of Internal Baculoviruses in Other *P. operculella* Populations

To find virus free *P. operculella* colonies, rearings based on individuals from Tunisia (TN) and Egypt (EG) were established and tested for the presence of persistent covert infections. PhopGV detection was based on *egt* type identification and was positive for the Tunisian (Phop-TN) and Egyptian (Phop-EG) laboratory population. Similar to Phop-IT a characteristic *egt* type II three band pattern was observed (Figure 9).

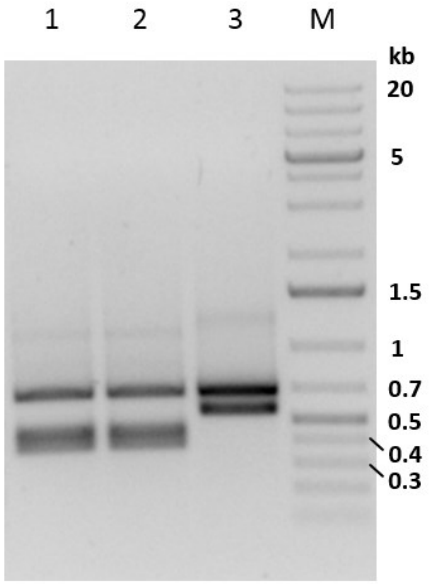

Figure 9. Electrophoresis through a 1% agarose gel of the *Alu*I digested PCR product *egt* from purified DNA of collected single larvae from the insect population Phop-TN (lane 1) and Phop-EG (lane 2) and a reference digest of an *egt* group III (PhopGV-GR1.1) (lane 3). M = GeneRuler 1 kb plus DNA ladder (Thermo Scientific). The gel image was inverted for better visualization of the PCR fragments.

When DNA of *P. operculella* was isolated from eggs, pupae or adults and applied to PCR analyses, a negative result was obtained (data not shown). A detection of PhopGV was only possible for the larval stages.

Discussion

Persistent covert infections are supposed to be a viral strategy for transmission and the answer to fluctuating host populations (Burden et al., 2003; Cooper et al., 2003). Persistent covert baculovirus infections had been observed not only for lepidopteran laboratory colonies but also in field populations (Vilaplana et al., 2010; Burden et al., 2003). In this study a stable covert PhopGV infection was detected, inside three laboratory reared insect populations of *P. operculella* (Phop-IT, Phop-TN and Phop-EG). The Phop-IT colony was the main rearing, the other two additional insect rearings were established in order to find a virus free *P. operculella* colony. The question if a completely virus free *P. operculella* colony is existing in general or every colony bears a potential virus infection cannot be answered certainly. There are theories that persistent infections cannot be cured and are likely the result of surviving virus challenge (Vilaplana et al., 2010; Cory and Myers, 2003; Andrealis, 1987). It is possible that covert virus infections are the rule and not the exception for insect populations. Occasionally such a covert infection may become overt. A crowding effect was described in the literature for being a stressor for covert infected insect colonies to start an active overt infection in *Trichoplusia ni* populations (Fuxa, 1999), *Colias philodice eurytheme* and *Junonia coenia* (Steinhaus, 1958), *Mamestra brassicae* (Burden et al., 2006; Hughes et al., 1993) and *Thaumatotibia leucotreta* (Opoku-Debrah et al., 2013).

There was no significant connection to crowding of insects and a triggered overt infection for PhopGV-IT, though there was a weak increase of virus outbreak with increasing number of larvae per potato disc. During the crowding experiment virus-caused mortality stayed below 5%, but there was a significant reduction in the total number of living individuals if low or high density number of individuals were put per potato disc. This could not be clearly assigned to virus activity because at high larval densities a food competition could also have an effect on the larval development. The pupation rate was significantly reduced at higher densities compared to low densities of Phop-IT larvae per disc. This reduction could be driven by either virus influence, food competition or stress at higher population densities.

The activation of covered infections to overt infections by infection with a second virus are reported for different baculoviruses; the activating virus can be either homologous or heterologous, belonging to another baculovirus species (Murillo et al., 2011; Burden et al., 2003; Cooper et al., 2003; Hughes et al., 1997 and 1993; Kelly et al., 1981). Such a triggering effect by a homologous virus isolate was also observed for PhopGV in this study when individuals of Phop-IT were infected by PhopGV-CR3 and the internal virus PhopGV-R was replicated at a high level. This effect was first noticed by the presence of the *egt* type II, when PhopGV-CR3 was propagated; the original *egt* type III of PhopGV-CR3 was only present in a minority of the propagated PhopGV-CR3.1. The phylogenetic reconstruction confirmed this assignment, whereas PhopGV-CR3.1 is clustering together with PhopGV-R.

Surprisingly, PhopGV-GR1 didn't activate the internal virus PhopGV-R during propagation in Phop-IT larvae. The resulting PhopGV-GR1.1 was free of contamination with the internal virus. Even when larvae were inoculated with a 1:1 mixture of PhopGV-R and PhopGV-GR1.1, only PhopGV-GR1.1 was detected in the progeny. This finding evidenced that not every homologous infection activates the internal virus in the Phop-IT population. In contrast, there must be a mechanism blocking the replication of PhopGV-R when larvae become infected with PhopGV-GR1 (superinfection exclusion).

Analyses of ORF 54 (*sod*) showed that PhopGV-R is composed of at least two different genotypes. This observation raises the question if both genotypes play a role in the covert infection of Phop-IT? It could be possible that the genotype present at the higher ratio of 91% is more virulent than the minor one and that selection of that genotype starts if the infection gets overt. In contrast, PhopGV-GR1 has only one *sod* type belonging to the same type as the majority genotype of PhopGV-R. Whether *sod* can be used as a selection marker for virulence, was not demonstrated yet and needs further investigations on its real function during the infection process.

All SNPs specific for PhopGV-R occurred either in majority or minority in PhopGV-CR3.1 (Table 2). This can be explained by the composition of PhopGV-CR3.1, consisting of 80% PhopGV-R. The finding that PhopGV-CR3.1 showed eleven SNPs in the majority and ten in the minority of reads can be explained by the instance that PhopGV-R is heterogenic and PhopGV-CR3.1 consists of at least three different genotypes in mixture.

For alphabaculoviruses it was reported, that super-infection exclusion is connected with actin reorganization (Beperet et al., 2014). It is still unclear, which mechanism is responsible for super-infection exclusion in connection with PhopGV isolates. Vertically transmitted pathogens may prevent super-infection (Lively et al., 2005), but the primarily vertical transmitted PhopGV-R did not show a super-infection exclusion. Rather PhopGV-R was excluded by the horizontally transmitted PhopGV-GR1. In contrast to super-infection exclusion, virus co-infections can occur in single larvae (Espinel-Correal et al., 2010; Cory et al., 2005; Smith and Vlak, 1988). A co-infection of single Phop-IT larvae was observed for PhopGV-R and PhopGV-CR3. It appears that PhopGV isolates interact in different ways if occurring in the same host individual, some PhopGV isolates exclude each other, whereas others tolerate each other.

Peroral infection experiments of *P. operculella* using low and high virus concentrations showed that the Phop-IT larvae became infected with both PhopGV-R and PhopGV-GR1, resulting in a low pupation rate (Figure 8). When PhopGV-R and PhopGV-GR1 were mixed, no significant reduction of the pupation rate under low virus pressure was observed. This may be explained by the suppression of isolate PhopGV-R and that only half of the amount of OB of PhopGV-GR1.1 caused an active infection compared to the single virus applications. At high virus concentration, each virus application, PhopGV-R or PhopGV-GR1 alone or mixture of both viruses, blocked pupation with a pupation rate close to 0%. This finding was similar to the observation of Sporleder et al. (2005) that *P. operculella* larvae infected with PhopGV completed larval development but failed to pupate, thus preventing development of further generations. Larval death and/or missing pupation of host larvae is common for baculovirus infections with GVs (Azam et al., 2016; Mukawa and Goto, 2008; Nakai et al., 2004 and 2002) and NPVs (Magholi et al., 2014; Burand and Park, 1992). One reason for the failure to pupate is that baculoviruses regulate host development through the activity of the virus-encoded *ecdysteroid UDP-glucosyltransferase* (*egt*) with the consequence that larvae are unable to molt or pupate (Nakai et al., 2016; Hughes, 2013; O'Reilly, 1995).

It seems to be a long-term survival strategy of some less virulent pathogens to adapt or manipulate the life cycle of the host (Moore, 2002). Highly virulent parasites and pathogens, on the contrary, are in risk to become extinct ('fade-out'), according to ecological models, because of a rapidly exhaustion of the pool of susceptible hosts (Earn et al., 1998, Swinton et al., 1998; Keeling and Grenfell, 1997). For insects with low densities overt baculovirus infections are rare (Myers, 1988) and horizontal virus transmission may be inefficient because of the low likelihood that a non-infected larva encounters an infected one (Anderson and May, 1981). If the virus becomes exposed to UV radiation outside the host, the infectivity of the virus can be reduced 10- to 100-fold within a few days (Jones et al., 1993;

Biever and Hostetter, 1985). Considering UV inactivation of OB and low host densities, selection for low virulence may increase the survival chance of the virus population, leading to persistent vertically transmitted covert baculovirus infections.

Insect communities, including *P. operculella,* may carry covert baculovirus infections which influence the host population dynamically through interaction of endogenous and exogenous viruses, either by tolerating, mixing or blocking each other when larvae are perorally infected. This finding needs to be considered to exploit the full potential of baculovirus application for plant protection reasons. It can be speculated that such covert infections of insect populations may contribute to the success and failure of baculovirus applications in the field, depending on the nature of interaction between the covertly present virus and the applied virus genotype, as observed in this study for the two isolates PhopGV-CR3 and PhopGV-GR1.

Chapter V

Interaction of Phthorimaea operculella granulovirus with a Microsporidium (*Nosema* sp. Phop) in Larvae of *Phthorimaea operculella*

Abstract

An antagonistic effect of a microsporidium (*Nosema* sp.) infection on the virulence of Phthorimaea operculella granulovirus (PhopGV) was recorded in mixed infected larvae of potato tuber moth (*Phthorimaea operculella*). When the *P. operculella* colony was infected at a high rate (42.8-100%) with the microsporidium, it was less susceptible to the isolate PhopGV-GR1.1. A 189,000 times higher virus concentration was necessary to cause the same level of mortality compared to the *P. operculella* colony when it was uninfected or had a low level of infection with the microsporidium (0-30%). This antagonistic effect was driven by a potentially new *Nosema* species (*Nosema* sp. Phop) which could be purified from microsporidian infected *P. operculella* individuals. The purified *Nosema* sp. Phop was characterised by morphological features, like size, filament coils and different developmental stages using transmission electron microscopy (TEM). On the molecular level, the partial cistron rDNA information of the small ribosomal subunit (SSU), the internal transcribed spacer (ITS), and the large ribosomal subunit (LSU) were identified. Phylogenetic analyses revealed that the newly described microsporidium belongs to the "true *Nosema*" clade; it was named *Nosema* sp. Phop. Partial sequence information of the RNA polymerase II largest subunit (RPB1) suggested that *Nosema bombycis* is the closest relative (98% identity). Interactions of microsporidia and betabaculoviruses are rarely described in literature, though mixed infections of different pathogens seem to be rather common events, ranging from antagonistic to mutualistic interaction. The observed antagonistic relationship between a potentially new *Nosema* sp. Phop and PhopGV-GR1.1 showed that pathogen interactions need to be considered when single pathogens are applied to insect populations, *e.g.* in the context of biological control of insect pests.

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Introduction

The potato tuber moth *Phthorimaea operculella* (Zeller) is a member of the lepidopteran family Gelechiidae and its larvae are considered as serious agricultural pests, especially in potato fields and storage in more than 90 countries worldwide (Kroschel and Sporleder, 2006). Besides potato (*Solanum tuberosum* L.), a number of other solanaceous plants are damaged by *P. operculella, e.g.* tomato (*Lycopersicon esculentum* L.), capsicum (*Capsicum anuum* L.), eggplant (*Solanum melogena* L.) and tobacco (*Nicotiana tabacum* L.) (Lacey and Kroschel, 2009). The insect's importance is rising due to global warming. In addition to tropical and Mediterranean regions, moderate and temperate regions are increasingly being colonized by this insect pest (Sporleder et al., 2008, 2004). The distribution of *P. operculella* is impaired by the activity of pathogens like nematodes, fungi, bacteria, protista or viruses; that reduce population numbers as well as the degree of damage caused by this insect (Haase et al., 2015; Lacey and Kroschel, 2009; Koppenhöfer, 2007; Hafez et al., 1997; Kroschel and Koch, 1996; Allen, 1954). The interaction of two pathogens of *P. operculella*, namely the Phthorimaea operculella granulovirus (PhopGV) (*Betabaculovirus*) and a microsporidium of the *Nosema* group, have been investigated in this study.

PhopGV is a natural pathogen of *P. operculella* that has already been used as a biological control agent against this pest in potato production and storage in Yemen and Latin America (Haase et al., 2015; Kroschel et al., 1996). It follows the typical infection process of a slow-killing granulovirus, with retarded development of infected larvae and tends to kill the infected host larvae in the final instar regardless of the instar in which the larvae were infected (Federici, 1997). This knowledge is crucial for the correct use as a biocontrol agent (Gómez Valderrama et al., 2017). Slow-killing viruses may have a high horizontal transmission efficiency and can suppress pest populations effectively in the long-term (Takahashi et al., 2015).

After *per os* ingestion of occluded virus particles (OBs) the surrounding granulin matrix of the OB is dissolved due to high pH (8-11) of the larval midgut and midgut epithelial cells become infected by the occlusion derived virions (ODVs) (Volkmann, 2008). Virus nucleocapsids are transported to the host cell nucleus where replication takes place, additional cells and whole tissues like fat body are infected via budded virus (BV) (Lacey et al., 2011). In the final stage of virus infection new OBs are produced by infected host tissues, followed by death of the host larvae and release of newly produced OBs to the environment, as infective source for other host individuals (Rohrmann, 2011).

Microsporidia are protists, and multigene phylogenies have revealed that they are close relatives to the kingdom of fungi (Solter and Becnel, 2012), building the superphylum Opisthosporidia, a deep-branch clade of Holomycota (Karpov et al., 2014; Keeling, 2014). They are opportunistic and obligate intracellular parasites with a spore size of 1-4 µm in diameter that cause chronic diseases and reduce the physiological and reproductive ability of their host (Corradi and Keeling, 2009). The coiled polar filament of microsporidian spores is used to inject the sporoplasm into the host cell upon spore germination. This polar filament and the feature of the diplokaryotic arrangement of the nuclei (in many species) clearly define microsporidia structurally (Vossbrinck and Debrunnner-Vossbrinck, 2005). Microsporidia can infect many different groups of vertebrate (including humans) and invertebrate hosts (Vavra and Lukes, 2013; Solter and Becnel, 2012; Canning, 1990). Many microsporidia infect arthropods, especially insects from the order Lepidoptera and Coleoptera (Corradi and Keeling, 2009; Weiser, 2005; Solter and Maddox, 1998). Chronic infection of a host causes prolonged developmental time. Adult size, longevity, fecundity, mating success and egg fertility can be reduced and mortality can be increased in all developmental stages (Novotny and Weiser, 1993). For example, microsporidia are considered as important cofactor in maintaining

population densities of the gypsy moth *Lymantria dispar* to a low level for a long period of time (Novotny, 1988; Weiser and Novotny, 1987). But only the microsporidium *Nosema locustae* has been registered as a biocontrol agent against locusts and grasshoppers in grasslands (Solter and Maddox, 1998).

Interactions between entomopathogens can be independent, antagonistic or mutualistic (Bauer et al., 1998). Mixed infections with baculovirus and microsporidium were already reported for other host pathogen systems, *e.g.* cadavers of *L. dispar* revealed a high prevalence of mixed viral and microsporidian infections (Novotny and Weiser, 1993; Weiser, 1987). Further reports of co-infections with a virus and a microsporidium are available for a number of different hosts from the Noctuidae and Apidae families with *Hyphantria cunea, Heliothis zea*, *Agrotis ipsilon*, *Spodoptera littoralis,* and *Apis mellifera* (Costa et al., 2011; Moawed et al., 1987; Cossentine and Lewis, 1984; Fuxa, 1979; Nordin and Maddox, 1972).

In the present study, a microsporidium infecting the laboratory rearing of *P. operculella* was identified as a *Nosema* sp. Phop. It was present also as mixed infections in larvae orally infected with PhopGV. The molecular and histopathological features of *Nosema* sp. Phop were characterized and the effect on host susceptibility to PhopGV was studied.

Material and Methods

Insects

A rearing of the potato tuber moth *P. operculella* was established at the Julius Kühn-Institut (JKI) in 2014. It was obtained from COOP. TERREMERSE, Bagnacavallo (Italy) and was based on insects collected in the Emilia Romagna, Ravenna. This strain of *P. operculella* was named Phop-IT. Rearing followed a changed protocol as described by Sporleder et al. (2005). Larvae were kept on potato slices at 26 °C and under 16/8 h light/dark photoperiod conditions until pupation. Potato slices were placed on sand to allow pupation outside of the potato and to ease the collection of the pupae with a mesh. After hatching, the adults were transferred to plastic cylinders (⌀=24.5 cm, h=18 cm) with open top and bottom. To avoid incoming light, the cylinders were lined with a dark plastic bag and put on a tray. The top end of the cylinder was covered with fine gauze that was penetrable for egg laying on a filter paper placed on top of the gauze outside of the cylinder. This technique allowed an exchange of the egg paper with a fresh one without opening of the cylinder. The adults were fed with 10% sucrose solution. After collecting the egg paper, the eggs were incubated at either 20 °C or 26 °C, to control the hatching day of the neonate larvae. Neonates were used for successive rearing cycles as well as bioassay experiments.

Baculovirus

The isolate PhopGV-GR1.1 originated from soil samples and was collected from a potato production area in Greece (Larem et al., submitted) in 2014. The obtained sample, PhopGV-GR1, was propagated in microsporidian-free *P. operculella* larvae, resulting in the passage named PhopGV-GR1.1. This propagated virus stock was used for all experiments.

Microsporidium

Individuals of Phop-IT showed an infection with a microsporidium, which was characterized as a *Nosema* species. This purified microsporidium obtained the working name "*Nosema* sp. Phop", indicating the host from which it was isolated. Screened larvae from the year 2015 showed no microsporidian infection. Microsporidian infected larvae were first observed in the population in February 2016. The infection rate increased thereafter, reaching close to 100% in the following months in 2017.

Microsporidian DNA Extraction from *P. operculella* Individuals

A modified protocol (Laarif et al., 2011) was used for microsporidian DNA extraction from potentially infected *P. operculella* individuals. One larva, pupa or adult was put into a 1.5 ml reaction tube together with 0.2 ml TE Buffer (pH 8) including 1% Triton-X 100 and was homogenized with a pistil. After incubation of 1 h at 65 °C, the suspension was centrifuged at full speed (20,800 × g) for 2 min. The DNA containing supernatant was used as the template for a PCR reaction or stored at -20 °C for later use.

PCR Amplification

For baculovirus detection, the oligonucleotide primer pairs egtF (5'-GAG TCG AGC CAA TTT TGT TTG CG-3') and egtR (5'-GCA ACG ATG ATC TCA TAT ATG AGC-3') (Jukes et al., 2014) were used for amplification of *ecdysteroid UDP–glucosyltransferase* (*egt*). Reaction comprised 10 µl 5 x Green Buffer, 1 µl dNTPs, 2 µl Primer forward and reverse (10 mM), 50 ng DNA template and 0.25 µl Go Taq Polymerase (5 U) (Promega GmbH, Mannheim, Germany). Reactions were made up to 50 µl using ddH_2O and the contents briefly centrifuged. The PCR protocol was: 94 °C for 4 min, followed by 33 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min.

The microsporidian detection was conducted with three oligonucleotide primer pairs for small subunit rRNA (SSU) 18f (5' -CAC CAG GTT GAT TCT GCC-3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3') (Weiss and Vossbrinck, 1999), RNA polymerase II second largest subunit (RPB1) MdRp1F1 (5'-AAG CCC GAT CTT AAT GCC ATT TGG-3') and MdRp1R1 (5'-GGC GTA ATC TTC TCT GGA AAC G-3') (Kyei-Poku and Sokolova, 2017) and internal transcribed spacer (ITS) ILSUf (5'-TGG GTT TAG ACC GTC GTG AG-3') and 530r (5'-CCG CGG KGC TGG CAC-3') (Hopper et al., 2016). The PCR protocol for SSU was: 94 °C for 2 min, followed by 35 cycles of 94 °C for 45 sec, 44 °C for 30 sec, 72 °C for 1.5 min and a final extension at 72 °C for 5 min. For RPB1 and ITS primer pairs an annealing temperature of 52 °C was selected.

Amplified products were visualised by 1% agarose gel electrophoresis at 90 V for 45 min in 1 x TAE buffer (40 mM Tris-acetate, 20 mM acetic acid, 1 mM EDTA) stained with Midori Green Advance (Nippon Genetics Europe GmbH, Düren, Germany). Visualisation and caption of the gel images was done with ChemoCam Imager ECL UV trans-illuminator and software (INTAS Science Imaging Instruments GmbH, Göttingen, Germany).

Virus OB Quantification

The OB concentration was determined using a Petroff Hausser counting chamber (depth 0.02 mm) (Hausser Scientific, Horsham, Pennsylvania, USA.) in the dark field optic of a light microscope (Leica, DMRBE, Leica Microsystems GmbH, Wetzlar, Germany). The concentration was calculated based on the mean of three independent countings of an appropriate dilution of the OB suspension of the PhopGV isolate. This procedure of virus titration was repeated before the beginning of every experiment.

Biological Activity of PhopGV Isolates

To determine the virulence in median lethal concentration (LC_{50}) of the PhopGV isolate, neonates of *P. operculella* were subjected to a full range bioassay using PhopGV suspensions to inoculate the surface of a potato disc (4.3 cm Ø and 0.5 cm thickness). By using a knife, parallel superficial incisions were cut into the potato discs. This treatment of the potato discs allowed larvae to stay in the scratches and facilitated the detection of the test larvae at the end of the bioassay for better evaluation. The surface of each potato disc was covered with 200 µl of virus suspension or with H_2O for the untreated control group. In pre-tests the susceptibility of the *P. operculella* larvae to PhopGV-GR1.1 was tested, to determine the range of virus treatments. The bioassay was performed in five ten-fold dilutions of OBs ranging from 5.1×10^6 to 5.1×10^2 OB/ml. These main interval concentrations were replicated three to eight times including at least three independent repetitions. Additionally, ten intermediate concentrations, ten-fold dilutions of 1.85×10^6 to 1.85×10^2 OB/ml, 1×10^4 to 1×10^2 OB/ml and 3×10^3 to 3×10^2 OB/ml were tested once for a better fit of the concentration-effect curve. Less susceptable individuals were treated in main intervals only, six ten-fold dilutions ranging 5.1×10^8 to 5.1×10^3 OB/ml in five replicates including three independent repetitions. Twenty neonate larvae were placed on each potato disc using a fine brush. Each of the potato discs was kept in a Petri dish and incubated at 26 °C and 16/8 h light/dark photoperiod. Mortality data were determined after 14 days by collecting the surviving larvae and pupae. The concentration response graphs including LC_{50} values and confidential limits were calculated with ToxRat Standard software (ToxRat Standard Version 3.2.1, ToxRat Solutions GmbH, Alsdorf, Germany). Relative potency of the two LC_{50} values was calculated referring to Finney (1964) using the formula $\log(\text{relative potency}) = \log(LC_{50\ \text{low microsporidia}}) - \log(LC_{50\ \text{high microsporidia}})$.

Screening of *P. operculella* Larvae for Microsporidian Infection

To quantify the microsporidian infection rate, a minimum of 50 *P. operculella* individuals of each test group were investigated microscopically. The larvae were randomly collected on day 14 after the evaluation of the associated bioassay and kept at -28 °C until disection. Preparation of fat body and midgut was done by opening the larval body from the abdomen towards the direction of the head, using a fine pair of scissors and tweezers. Each tissue type was analysed separately after transferring it to a microscope slide. Larvae were screened for PhopGV and microsporidia infection using phase contrast microscopy (400 x magnification) (Leica, DMRB, Leica Microsystems GmbH, Wetzlar, Germany).

TEM Preparation

Microsporidian infected *P. operculella* larvae were fixed in Karnovsky fixative (5% glutaraldehyde, 4% paraformaldehyde in 0.2 M cacodylate buffer pH 7.4) at 4 °C for 12 h. Midgut and fat body were removed and incubated in Karnovsky fixative for another 48 h at 4 °C. The samples were washed three times for 30 min in 0.1 M sodium cacodylate buffer ($Na(CH_3)_2AsO_2$ x $3H_2O$), incubated in 2% OsO_4 in 0.1 M sodium cacodylate buffer for 46 h and washed three times for 1 h in sodium cacodylate buffer inclusive 2.5% sucrose. Contrast staining was done with phosphotungstic acid uranyl acetate (5 h) followed by dehydration (EtOH 70% (1 h), 80% (1 h), 96% (2 x 1 h) and 100% (2 x 1 h)). The embedding was done in propylene oxide and Spurr (Siegma-Alderich, St. Louis, Missouri, USA). The dehydrated samples were first put into propylene oxide (2 x 1 h), followed by increasing Spurr ratio to substitute the propylene oxide: Propylene oxide - Spurr 4:1 (1 h), 3:1 (1 h), 1:2 (over-night), 1:3 (4 h), 1:4 (4 h) and Spurr alone (over-night). For hardening, the samples were heated to 70 °C for 8 h. Thin sections of 50 nm were prepared using an ultra-microtome (Reichert Ultracut S; Leica Microsystems GmbH, Wetzlar, Germany), followed by contrast staining with uranyl acetate for 10 min and washing with double distilled water. The stained sections were treated with lead citrate for 2 min using NaOH pellets to fix the CO_2 from the surrounding air to avoid precipitation on the samples. Finally, the samples were washed with double distilled water. Thin sections were investigated with a transmission electron microscope (TEM 902, ZEISS, Oberkochen, Germany).

Sequencing and Phylogeny

DNA samples from two different microsporidian infected *P. operculella* individuals were used for PCR with SSU (partial), ITS (+LSU partial) and RPB1 (partial) primer pairs. The obtained PCR products were Sanger sequenced (Seqlab-Microsynth, Göttingen, Germany). PCR fragments were sequenced in forward and reverse direction and a consensus sequence was generated from a sequence coverage of four nucleotides per position. All consensus sequences were compared with the database of the National Center for Biotechnology Information (NCBI, U.S. National Library of Medicine) by BLASTN to confirm that the sequencing result was microsporidian derived and no other DNA contamination was sequenced.

MUSCLE 3.6, using the mauve aligner algorithm (Darling et al., 2004) of the program Geneious 10.0.5, was applied to generate alignments for the 1,164 bp SSU (partial sequence), 277 bp ITS (+ LSU partial sequence) with sequences of 24 *Nosema*- and one *Vairimorpha* species available on GenBank (Table 1). The single alignments as well as the concatenated alignments with a total length of 1,441 bp were imported to MEGA6 (Tamura et al., 2013) and the best DNA model for phylogenetic reconstruction was calculated from all available maximum likelihood algorithms including gamma prediction and invariant sites. For every phylogenetic tree reconstruction, partial deletions, a site coverage cutoff (95%), 500 bootstrap replicates and Nearest-Neighbor-Interchange (NNI) were applied.

Table 1. Accessions and hosts of 25 microsporidian species (24 *Nosema* sp. and one *Vairimorpha* sp.) used for phylogenetic analysis. Asterisks (*) indicating samples for those with no information about the host species from which the sample was originally isolated. A possible host is indicated in brackets.

Accession	Species name	Host		
		Order	Family	Species
AY741111.1	*N. bombi*	Hymenoptera	Apidae	*Bombus terrestris*
U97150.1	*N. apis*	Hymenoptera	Apidae	*Apis mellifera*
DQ078785.1	*N. ceranae*	Hymenoptera	Apidae	(*Apis cerana*)*
DQ486027.1	*N. ceranae*	Hymenoptera	Apidae	*Apis mellifera*
EU338534.1	*N.* sp.	Lepidoptera	Pieridae	*Eurema blanda arsakia*
AY383655.1	*N.* sp. C01	Lepidoptera	Pieridae	*Pieris rapae*
HQ399665.1	*N.* clone MPr	Lepidoptera	Pieridae	*Pieris rapae*
HQ891818.1	*V.* sp. sd	Lepidoptera	Bombycidae	*Bombyx mori*
AY209011.1	*N. bombycis*	Lepidoptera	Bombycidae	(*Bombyx mori*)*
KM190863.1	*N.* sp. PM-1	Lepidoptera	Papilionidae	*Papilio machaon*
FJ772435.1	*N. heliothidis*	Lepidoptera	Noctuidae	*Helicoverpa armigera*
AY747307.1	*N. spodopterae*	Lepidoptera	Noctuidae	(*Spodoptera litura*)*
KT336240.1	*N.* sp. SE	Lepidoptera	Noctuidae	*Spodoptera exigua*
JN882299.1	*N.* sp. HA	Lepidoptera	Geometridae	*Hemerophila atrilineata*
HQ457431.1	*N. disstriae*	Lepidoptera	Lasiocampidae	*Malacosoma disstria*
HQ457432.1	*N. fumiferanae*	Lepidoptera	Tortricidae	*Choristoneura fumiferana*
HQ457434.1	*N.* sp. CO 05-11	Lepidoptera	Tortricidae	*Choristoneura occidentalis*
HQ457433.1	*N.* sp. CPP 04-02	Lepidoptera	Tortricidae	*Choristoneura pinus pinus*
AY960986.1	*N.* sp. PX-1	Lepidoptera	Plutellidae	*Plutella xylostella*
AY960987.1	*N. plutellae*	Lepidoptera	Plutellidae	*Plutella xylostella*
DQ073396.1	*N. antheraeae*	Lepidoptera	Saturniidae	*Antheraea pernyi*
KP100640.1	*N.* sp. HR	Lepidoptera	Papilionidae	*Histia rhodope*
FJ767862.1	*N.* sp. SC	Lepidoptera	Saturniidae	*Samia cynthia ricini*
This work	*N.* sp. Phop	Lepidoptera	Gelechiidae	*Phthorimaea operculella*
FJ969508.1	*N.* sp. PA	Coleoptera	Chrysomelidae	*Phyllobrotica armata*
KY783624.1	*N. maddoxi* clone 9	Hemiptera	Pentatomidae	*Chinavia hilaris*

Results

Biological Activity of PhopGV-GR1.1 in Healthy and Microsporidian Infected Larvae

All PhopGV-GR1.1 treated samples showed the presence of virus occlusion bodies (OB) in the midgut and fat body tissue, but some individuals showed an additional co-infection with microsporidia in the same tissues. Control insects showed either no infection or microsporidia infections only. The concentration mortality response of PhopGV-GR1.1 infected larvae was determined for two groups of the same *P. operculella* strain, with ≤ 30% prevalence and ≥ 42% prevalence of microsporidia infection, resulting in LC_{50} values of 2.17×10^3 OB/ml and 1.59×10^8 OB/ml respectively (Figure 1, Table 2). A total number of 2,575 of the ≤ 30% prevalence group and 600 individuals of the ≥ 42% prevalence group were tested each in more than three independent replicates. The concentration mortality data of the low rate infected individuals was combined to the so called ≤ 30% prevalence group, because there was no differing effect visible for populations infected with microsporidia in that range (Figure 1a). An increased infection rate above 42.8% microsporidian infected individuals in the population led to a shift in the virus concentration mortality relation of *P. operculella* larvae to the right (Figure 1b). A virus induced mortality of 100% of the infected larvae was reached under low microsporidia pressure (Figure 1a), but under high microsporidia pressure the mortality did not exceed 55% (Figure 1b).

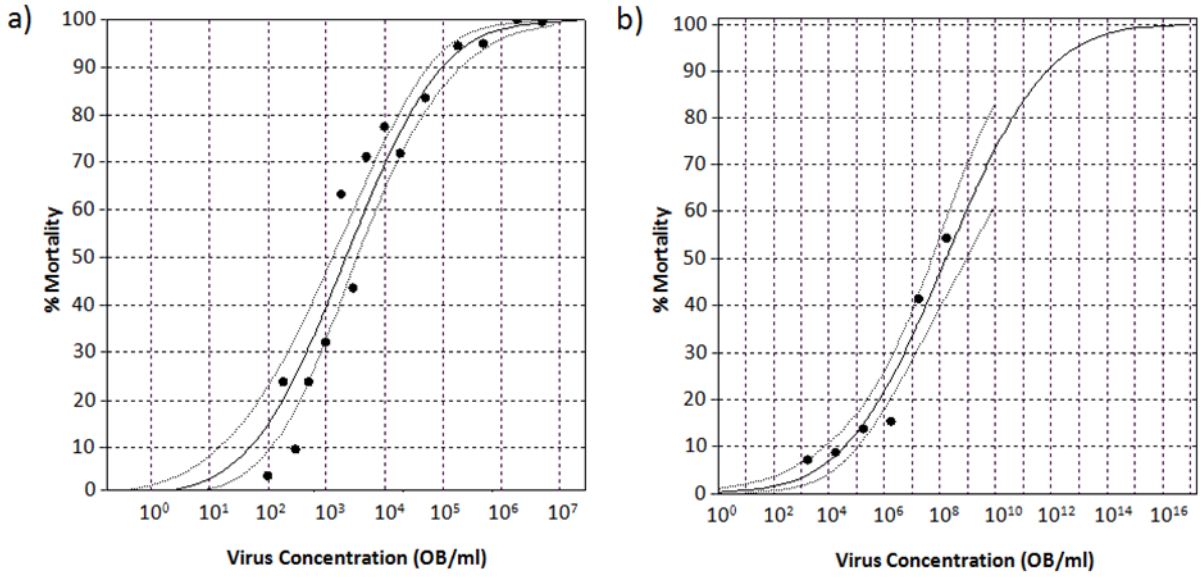


Figure 1. Concentration-mortality response of Phop-IT larvae after 14 days post infection (dpi) with different concentrations of PhopGV-GR1.1 and zero to low microsporidian presence (a) and moderate to high microsporidian infection rates (b). Black dots represent the data points, black lines the function and pointed lines the 95% - confidential limits. The data was Abbott (1925) corrected.

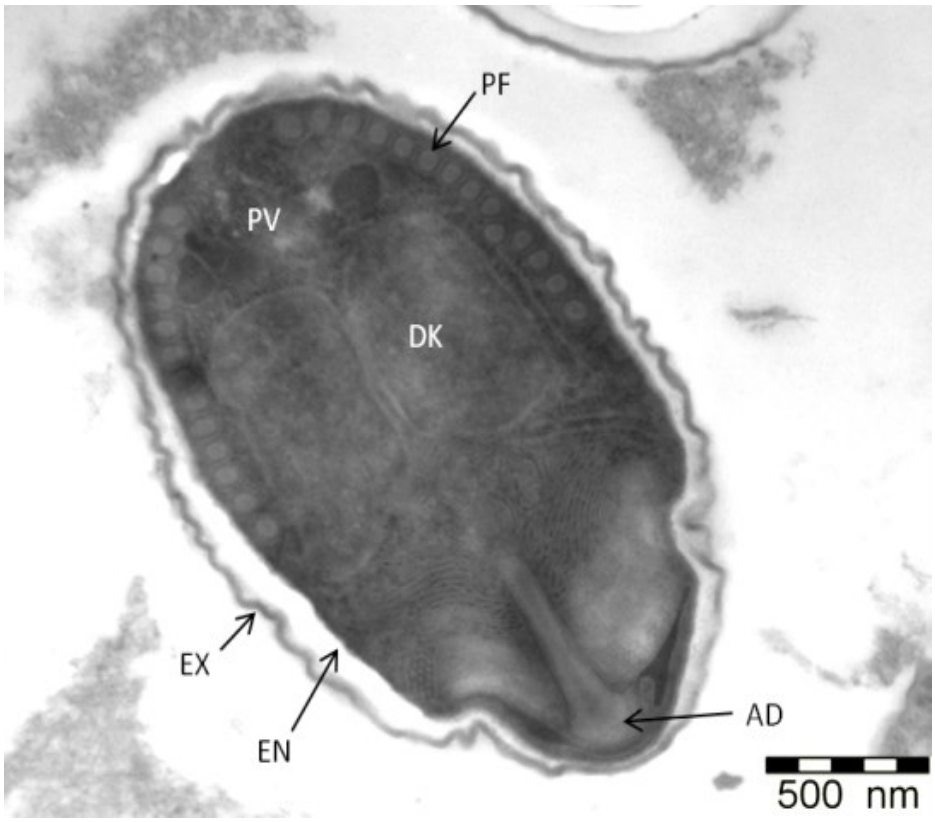
Table 2. LC_{50} values for PhopGV-GR1.1 and varying microsporidian presence in the host. Given are percentage of microsporidian infected individuals with the range of infection (%), the median lethal concentration (LC_{50}) in occlusion bodies per ml with 95% confidential limits, the relative potency, number of tested individuals (n), mortality (%) of the control group, the slope and chi square (Chi^2) with degrees of freedom (df).

Microsporidia [%] (Range)	LC_{50} [OB/ml] (95% conf. limits)	Relative Potency	n	Mortality (%) of Control	Slope	Chi^2 (df)
15.5 (0 - 30)	2.17×10^3 (1.29 - 3.37)	1	2,275	35.5	0.77	40.90 (13)
68.6 (42.8 - 100)	1.59×10^8 (0.5 - 8.61)	1.36×10^{-5}	600	23.3	0.35	6.79 (4)

Morphology of Microsporidian Spores

Infections with microsporidia were observed in all developmental stages of *P. operculella,* larvae (L1–L4), pupae and adults. Larvae were used for screening of infection, because this developmental stage was also used to perform the bioassays with PhopGV-GR1.1. If a microsporidian infection of a Phop-IT larva was observed, midgut and fat body showed a high number of mature spores (Figure 2), but other developmental stages like meronts, sporonts and sporoblasts were found as well (Figure 3). The posterior vacuole, anchoring disc, endo- and exospore were visible on thin sections of mature spores under transmission electron microscopy (Figure 2). Measuring of 180 spores resulted in an average spore length of 3.6 ± 0.01 µm (mean ± SE) and width of 2.0 ± 0.01 µm (mean ± SE). The spores showed an ovoid shape and a diplokaryon. Polar filament coils were in line with an isofilar arrangement of 12 ± 1 (min/max = 11/16, n = 50) polar filament coils in a row. In 84% of 50 counted spores the polar filament built a single row, the remaining 16% showed an arrangement with a second row. The polar filament was connected to the anterior of the spore with the anchoring disc (Figure 2a), which penetrates the host cell membrane when the polar filament is extruded. Mature spores showed a thick translucent endospore wall and a thin electron dense exospore wall (Figure 2b). The polaroplast has a distinctive granular structure, an isofilar polar filament, a posterior vacuole, and rows of polyribosomes. The morphology of the analysed microsporidium was typical for *Nosema* (Terry et al., 1999; Nordin and Maddox, 1974). This microsporidium was thus termed *Nosema* sp. Phop.

a)



b)

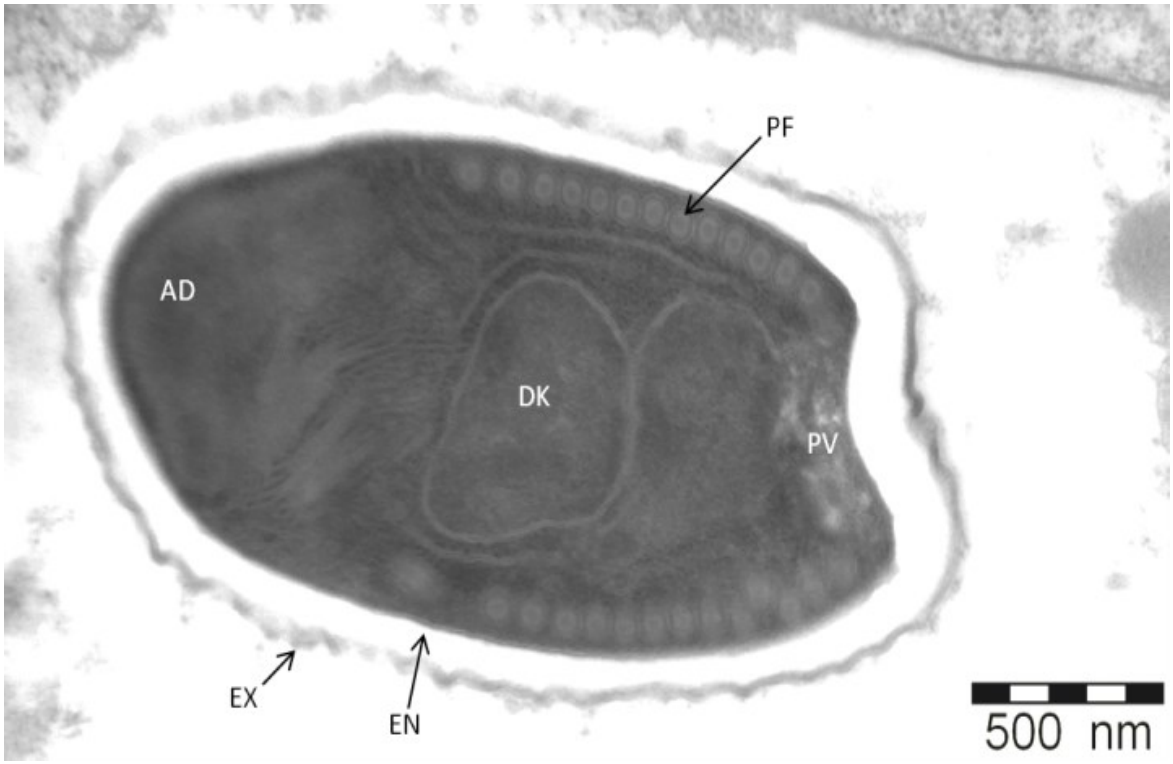


Figure 2. Transmission electron micrographs of *Nosema* sp. Phop spores. (a) Section through the anchoring disc. (b) Mature spore with a thick endospore wall in the midgut tissue of the host *Phthorimaea operculella*. AD = anchoring disc; EX = exospore; EN = endospore; DK = diplokaryon; PF = polar filament; PV = posterior vacuole.

Life Cycle of *Nosema* sp. Phop

Meronts were diplokaryotic with a thin plasma membrane surrounding cytoplasm and the diplokaryon. A double nuclear membrane enclosed both individual nuclei, each having a darkened area rich in chromatin (Figure 3a). Sporonts could be distinguished from meronts by a thickened plasma membrane and an irregular shape. Both show a diplokaryotic nucleus, endoplasmic reticulum and ribosomes (Figure 3b). Matured sporonts form sporoblasts have a more regular shape (Figures 3c, d) and start to build organelles like polaroplast (Figure 3c) and polar filament (Figure 3d). A differentiation of the plasma membrane to endospore and exospore walls and developed organelles define the spore stage of the microsporidian life cycle (Figure 2).

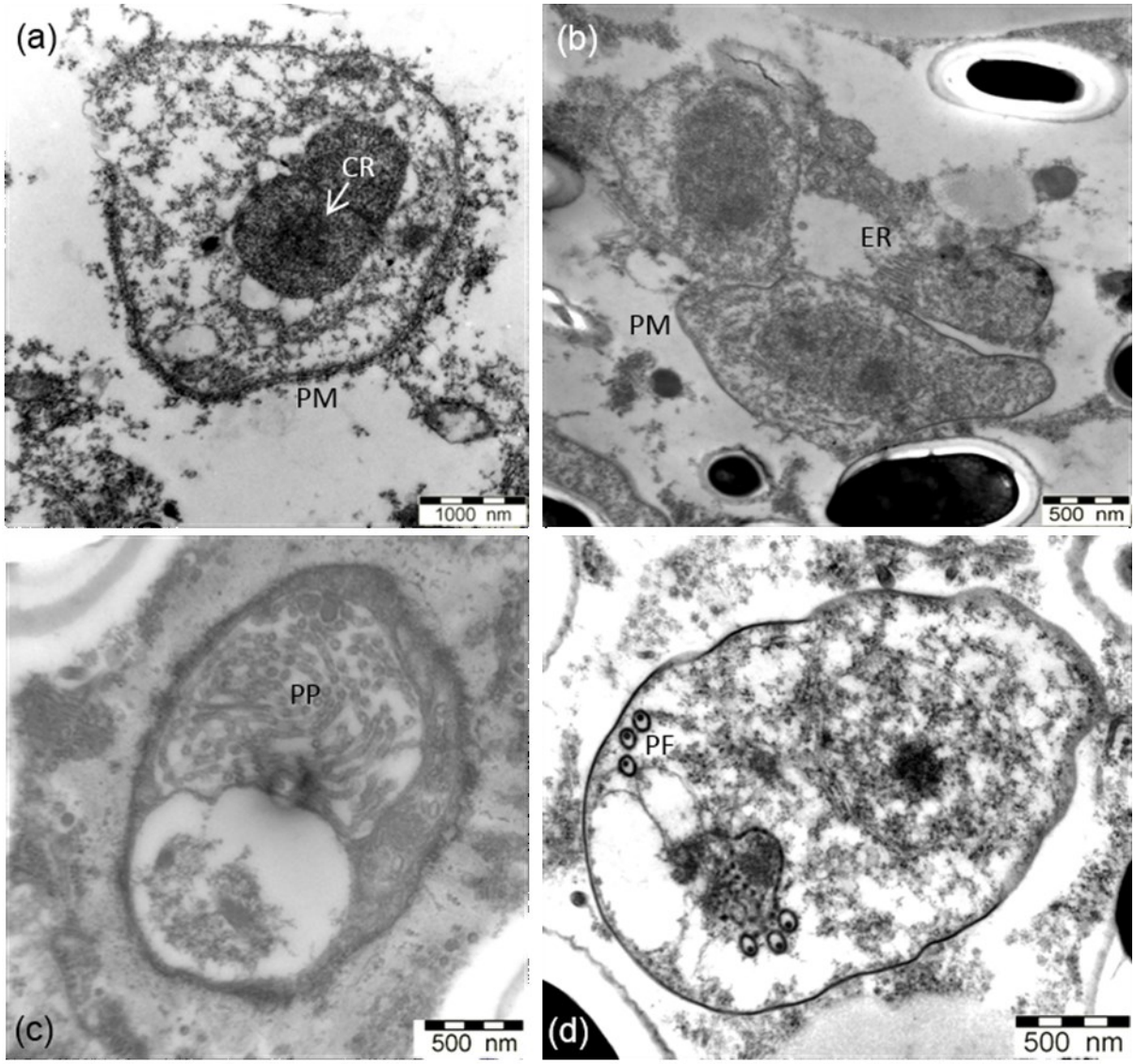


Figure 3. Transmission electron micrographs of different developmental stages of *Nosema* sp. Phop. (a) Meront with a thin plasma membrane (PM) surrounding nuclei and cytoplasm. The diplokaryon is chromatin (CR) rich. (b) Paired sporonts, which can be recognized by thickening plasma membrane (PM) and endoplasmic reticulum (ER). (c) Sporoblast with a forming polaroplast (PP). (d) Sporoblast with forming polar filament (PF), organelles and a beginning formation of cell wall structures.

Phylogenetic Reconstruction Based on *Nosema* sp. Phop Partial SSU- and ITS (+ partial LSU)-rDNA

To determine the genetic relation of *Nosema* sp. Phop towards other microsporidia, PCR using specific oligonucleotide primers for partial SSU- and ITS (+ partial LSU)-rDNA resulted in fragments which could be sequenced and used for phylogenetic reconstruction. The first observation was the reverse order of the ribosomal subunits (LSU-ITS-SSU), typical for the "true *Nosema*" clade (Figure 4).

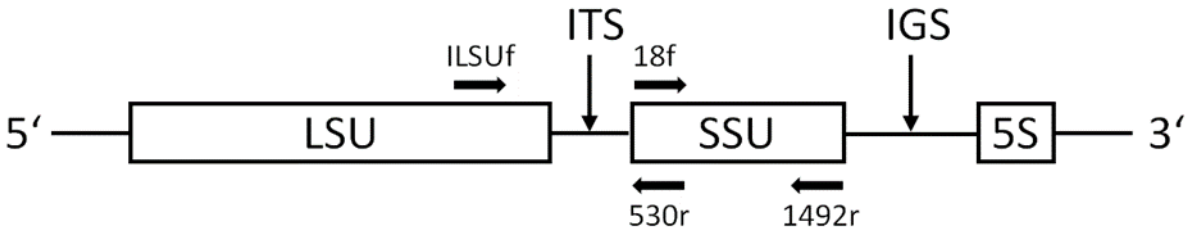


Figure 4. Schematic diagram of the arrangement of *Nosema* sp. Phop rRNA cistron, with corresponding PCR primers. The reverse order of rRNA subunits is typical for the "true *Nosema*" clade (5'-LSU-ITS-SSU-IGS-5S-3' (LSU, large subunit rRNA gene; SSU, small subunit rRNA gene; ITS, internal transcribed spacer; IGS, intergenic spacer; 5S, 5S ribosomal RNA). Diagram adapted from Kyei-Poku and Sokolova (2017).

Three phylogenetic reconstructions using 25 species (Table 1) were performed based on different parts of the rDNA of *Nosema* sp. Phop purified from *P. operculella* larvae. It has to be noted that all phylogentic analyses resulted in two separate clades with the "true *Nosema*" clade, formed around *N. bombycis,* and a second *Nosema/Vairimorpha* clade. *Nosema* sp. Phop was assigned in all analyses to the "true *Nosema*" clade (Figure 5, 6, 7). The three resulting trees were highly homogeneous in the separation of the two clades and the placement of the species with one exception: *Nosema* sp. C01 (AY383655.1) was assigned two times closer to the "true *Nosema*" clade (Figure 5, 7) and one time to the *Nosema/Vairimorpha* clade (Figure 6). Another *Nosema* species also isolated from *Pieris rapae* (*N.* clone MPr), however, was assigned in all analyses to the clade *Nosema/Vairimorpha*. The identity of the SSU sequence of *Nosema* sp. Phop with its most closely related neighbour *Nosema* bombycis (AY209011.1) was 100%. Same identity was reached for *Nosema* sp. Phop SSU sequence with *Nosema* sp. PM-1 (KM190863.1), *Nosema* sp. heliothidis (FJ772435.1), *Nosema* sp. spodopterae (AY747307.1) and *Nosema* sp. PX1 (AY960986.1).

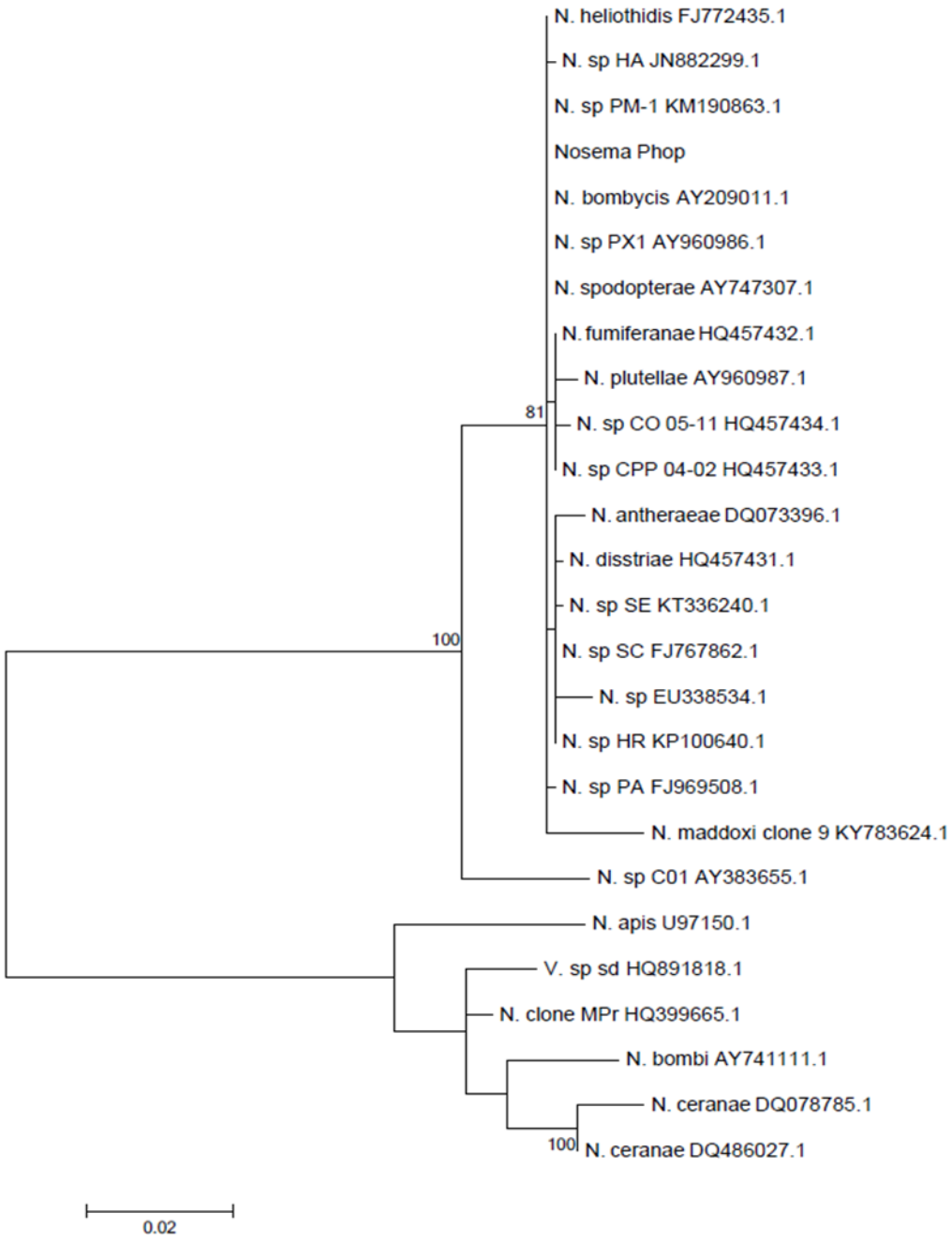


Figure 5. Molecular phylogenetic analysis by Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992) of the small ribosomal subunit (SSU) partial information of 1,164 bp aligned from 26 nucleotid sequences. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated with a bootstrap value of 500. There were a total of 1,007 positions in the final dataset. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 14.8647% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

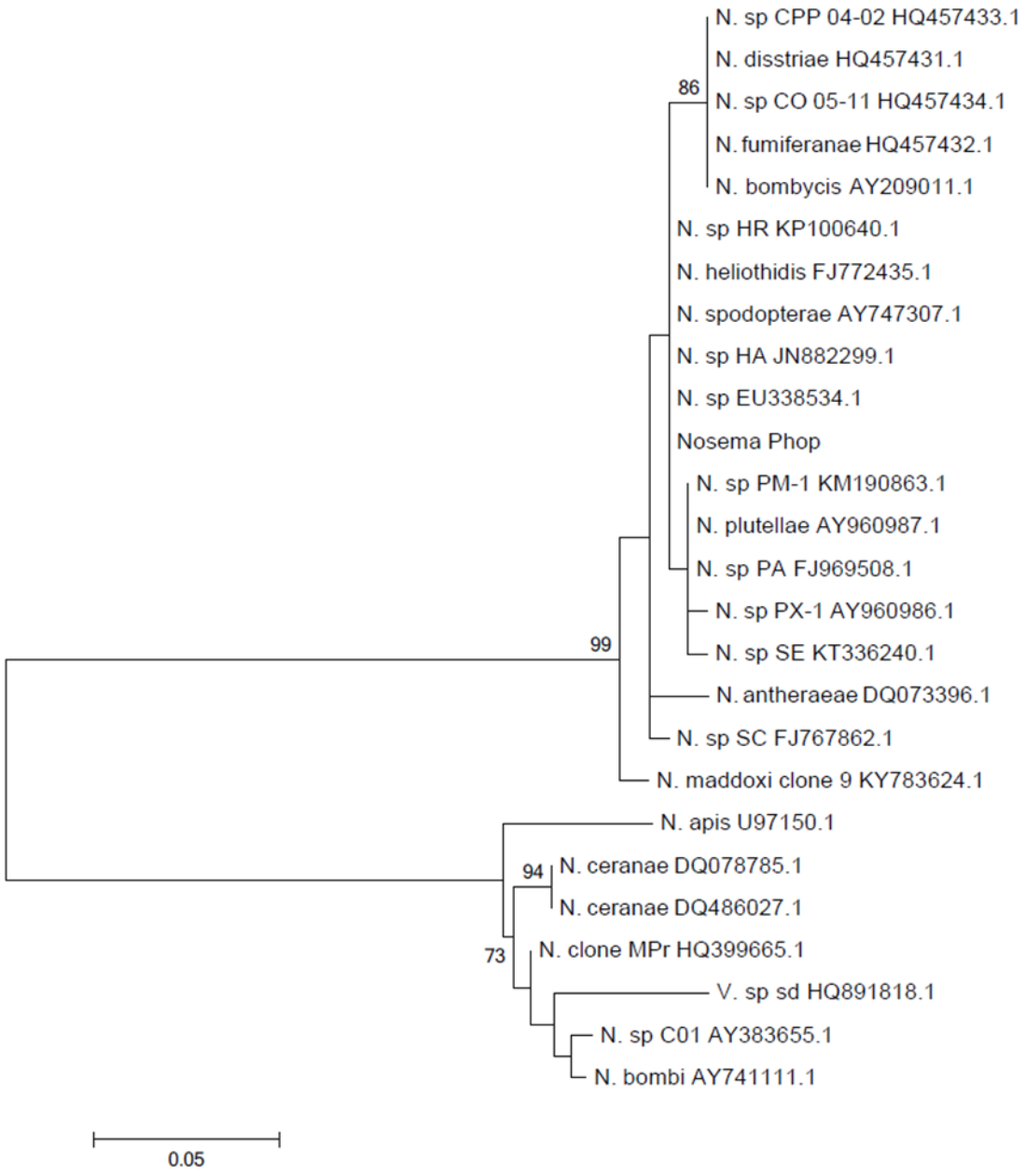


Figure 6. Molecular phylogenetic analysis by Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992) of partial information of internal transcribed spacer (ITS) and large ribosomal subunit (LSU) of 277 bp aligned from 26 sequences. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated with a bootstrap value of 500. There were a total of 203 positions in the final dataset. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+G, parameter = 0.2950)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 26.1799% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

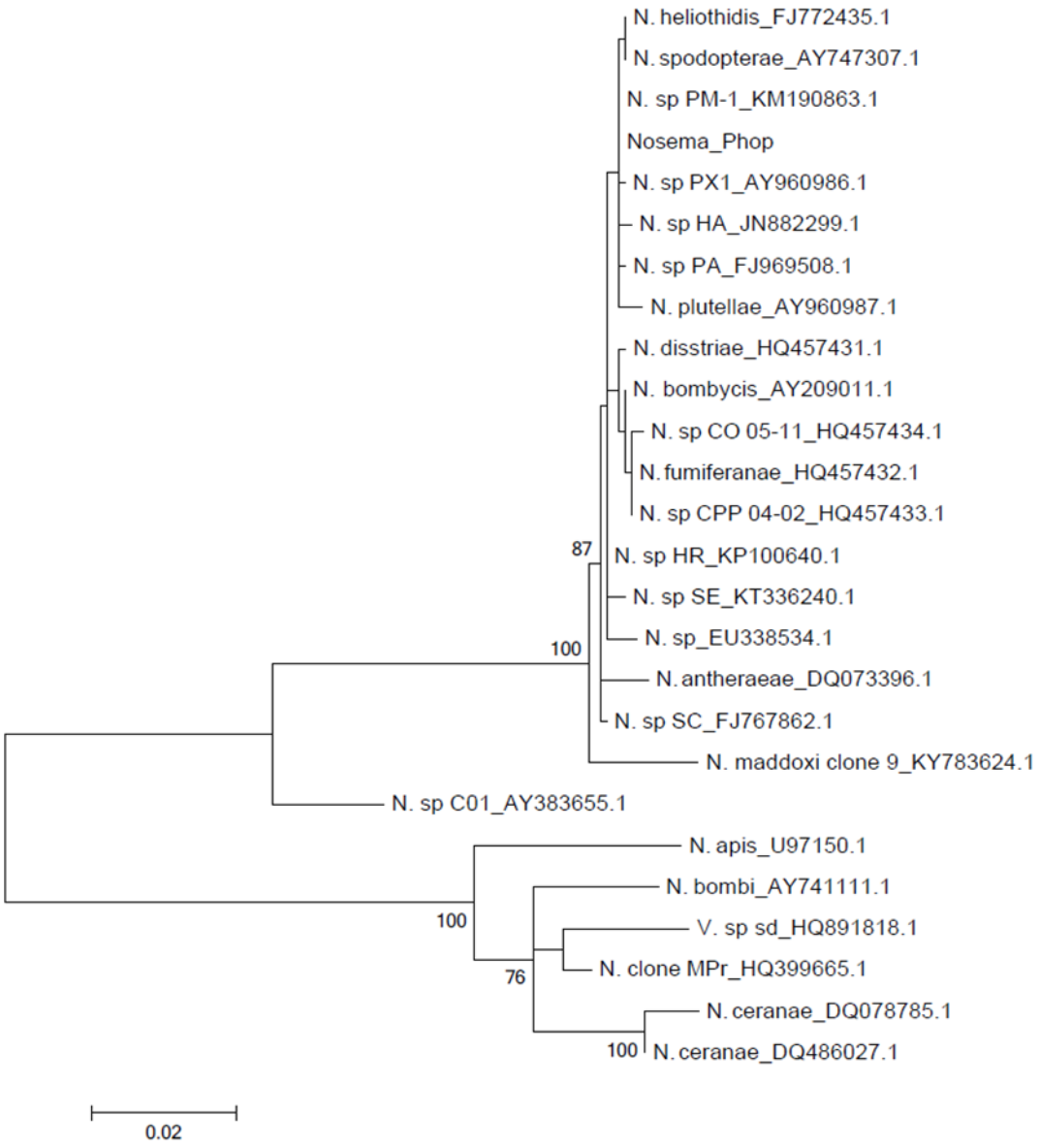


Figure 7. Molecular Phylogenetic analysis by Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) of SSU partial information, complete ITS and partial LSU of 1,441 bp aligned from 26 nucleotid sequences. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated with a bootstrap value of 500. There were a total of 1,242 positions in the final dataset. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 17.1704% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Comparison of RPB1 Isolated from *Nosema* sp. Phop to Closely Related Species

A 714 bp partial coding sequence of RNA polymerase II largest subunit (RPB1) was amplified by PCR from *Nosema* sp. Phop and sequenced. A BLASTN search with GenBank resulted in 14 hits for *Nosema* species from which half of the hits were *Nosema bombycis* with the best score value of 1,242 and an identity of 98% (Table 3). The other hits spread over seven different *Nosema* species, namely *N. tyriae, N. trichoplusiae, N.* sp. PA*, N.* sp. Cpp 04-02*, N. disstriae, N.* sp. CO 05-11 and *N. fumiferanae* (Table 3). This confirms the assignment of *Nosema* sp. Phop to the "true *Nosema*" clade, which is forming around *N. bombycis*.

Strikingly, one hit with a score value of 1,242 (query coverage 97%, E value 0.0, identity 98%) was found for the predicted RPB1 of the Lepidoptera *Papilio xuthus* (XM_013320129.1). Two other hits of the same species were found with lower score (1,203 and 1,181) and identity (97%) values (XM_012206384.1 and XM_013314171.1).

Table 3. BLASTN hits for partial coding sequence of RNA polymerase II largest subunit (RPB1) for *Nosema* sp. Phop.

Taxonomy	Number of hits	Number of organisms	Score (Max = Total)	Query Cover. (%)	E Value	Ident. (%)	Accession Number
Nosema	**14**	**8**					
- bombycis	**7**	**1**	**1,242** **1,208** **1,194** **1,194** **1,181** **1,170** **1,158**	**97** **97** **99** **99** **97** **97** **97**	**0.0**	**98** **98** **97** **97** **97** **97** **96**	**JX213754.1** **JX213755.1** **JX213751.1** **DQ996231.1** **JX213752.1** **JX213753.1** **JX213750.1**
- tyriae	**1**	**1**	**1,230**	**97**	**0.0**	**98**	**AJ278948**
- trichoplusiae	**1**	**1**	**1,181**	**97**	**0.0**	**97**	**DQ996234.1**
- sp. PA	**1**	**1**	**1,164**	**97**	**0.0**	**96**	**KJ728831.1**
- sp. CPP 04-02	**1**	**1**	**1,046**	**97**	**0.0**	**93**	**HQ457437.1**
- disstriae	**1**	**1**	**1,038**	**99**	**0.0**	**93**	**HQ457438.1**
- sp. CO 05-11	**1**	**1**	**1,029**	**97**	**0.0**	**93**	**HQ457436.1**
- fumiferanae	**1**	**1**	**1,002**	**97**	**0.0**	**92**	**HQ457435.1**

Discussion

Here the identification of a *Nosema* species purified from *P. operculella* belonging to the "true *Nosema*" clade and its correlation with the reduction of susceptibility of *P. operculella* larvae to PhopGV-GR1.1 was reported. The biological activity of PhopGV-GR1.1 against *P. operculella* larvae was largely reduced in the presence of the microsporidium *Nosema* sp. Phop. The LC_{50} value of PhopGV-GR1.1 against *P. operculella* was a factor of 10^5 higher if individuals of the host population were infected by *Nosema* sp. Phop in a high rate (48.8-100%). Compared to microsporidia-free larvae, the co-infected larvae were smaller in size, showed weak lethargic behaviour, but did not die as expected after baculovirus infection. This effect was most likely driven by microsporidian activity in infected host cells. Reduced larval size was reported after infection of neonate and third instar *Agrotis ipsilon* larvae with *Vairimorpha* sp. and *V. necatrix* that also led to less tissue mass for virus infection with Rachiplusia ou nucleopolyhedrovirus (RoMNPV), if a double infection took place (Cossentine and Lewis, 1984). Inhibiting apoptosis appears to be a common mechanism used by microsporidia (Del et al., 2006; Scanlon et al., 1999). For *Bombyx mori* cells, it has been reported that *N. bombycis* prevents apoptosis and reactive oxygen species (ROS) production to create the optimal environmental condition for its growth and development (He et al., 2015). Maybe the microsporidium attenuates the negative effects of a baculovirus infection to the host cell as well. Virus activity would destroy the infected cells, which would lead to death of the host and release of virus particles. This release of virus particles would take place earlier without the influence of the microsporidium. In case of a co-infection with virus and microsporidium, the host may die on a later stage despite an ongoing virus infection, to allow the microsporidium to complete its life cycle and reproduce before the death of the host. This may help to keep microsporidia infection in the population of *P. operculella*. Larvae, which are first infected with microsporidia and then become infected by virus will contaminate the environment with a mixture of spores and polyhedra (Bauer et al., 1998). This hypothesis is supported by the idea that many host-pathogen systems indicate that "concomitant" or "mixed" infections involving two or more parasite species or genotypes are common and may even be the rule (Thomas et al., 2003; Cox, 2001; Read and Taylor, 2001).

Time to death was significantly longer for *L. dispar* larvae infected only with microsporidia (*Nosema* sp.) compared to larvae infected with LdMNPV alone (Bauer et al., 1998). Antagonism is not unexpected as both pathogens, PhopGV and *Nosema* sp. Phop, compete for the same host tissues, midgut epithelia and fat body. Virus and microsporidia may compete for cellular resources or interact by changing cellular functions. A microsporidian infection lowered virus polyhedral production in co-infected *L. dispar* larvae (Bauer et al., 1998). Fuxa (1979) hypothesized antagonism between virus and microsporidium should have three reasons: 1) both pathogens penetrate the midgut epithelium by different methods and compete for the entry into the hemocoel; 2) competition for fat body cells, which both infect; and 3) metabolic interference. Varying degrees of competition between microsporidia and NPV are documented in a variety of lepidopterans (Moawed et al., 1987; Cossentine and Lewis, 1984; Fuxa, 1979; Nordin and Maddox, 1972; Lipa, 1971). In adult honey bees (*Apis mellifera ligustica*), an antagonistic interaction was reported between *Nosema ceranae* and deformed wing virus (DWV), if midgut cells were infected by both pathogens (Costa et al., 2011).

On the other hand, mutualistic effects were reported for black queen cell virus (BQCV) together with *Nosema apis* (Bailey and Ball, 1991; Bailey et al., 1983) and chronic bee paralysis virus (CBPV) together with *Nosema ceranae* (Toplak et al., 2013). Also for Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV) and *Nosema* sp. a mutualistic effect was reported when co-infection of *L. dispar* larvae took place, but the pathogenesis effect was variable, ranging from mutualistic to antagonistic, depending on the sequence of infection (Bauer et al., 1998).

The diplokaryotic nucleus, absence of an interfacial envelope or of regular spore groupings, and the diasporoblastic sporogony are all characteristics of the genus *Nosema* (Terry et al., 1999). Further, the reverse order of ribosomal subunits (LSU-ITS-SSU) and the blast of the partial sequenced SSU, LSU, ITS and RPB1 allowed assigning the microsporidium found in Phop-IT to the genus *Nosema*. The reversed arrangement of the ribosomal gene was first identified in *N. bombycis* (Huang et al., 2004) and further confirmed in at least nine additional species within the "true *Nosema*" clade, but not for any species of the "*Vairimorpha*" clade (Hajek et al., 2017; Kyei-Poku and Sokolova, 2017). The ssrDNA is frequently used to analyse the basic relationships among microsporidia, whereas RNA polymerase II gene (RPBI) is better for analysing microsporidia on the species level (Vossbrinck and Debrunner-Vossbrinck, 2005). Phylogenetic reconstruction was done using the cistron information. For the RPBI, fewer reference sequences were available on GenBank, probably because the RPBI has four subunits which makes it more complicated to purify and identify these subunits. Instead of using the RPBI information for phylogenetic reconstruction, the partial sequence against the GenBank available entries was compared. Many close related *Nosema* species were found but there was no complete match. Together with the phylogenetic reconstruction, it is assumed that there was purified a *Nosema* sp. of the "true *Nosema*" clade that was not described before. In all performed phylogenetic reconstructions *Nosema* sp. Phop was assigned together with *N. bombycis*. The RPBI analyses confirmed this result with *N. bombycis* as the best hit for *Nosema* sp. Phop. The BLASTN hits for the predicted RPBI of the lepidopteran *Papilio xuthus* were not further considered for this study because it is doubtful whether these sequences really originated from the Lepidoptera. It is more likely that they derived from an accidental sequencing of a *P. xuthus* infected with *Nosema*.

Further morphological characteristics like the size of the spores and the number of polar filament coils differentiated *Nosema* sp. Phop from other *Nosema* species, such as *N. fumiferanae*, *N. bombycis* and *N. ceranae* (Kyei-Poku and Sokolova, 2017; Hopper et al., 2016; Fries et al., 1996). A *Nosema* species (*Nosema* sp. PO) purified from the host *P. operculella* in Tunisia (Laarif et al., 2011) showed the same number of polar filament coils (12). But compared to *Nosema* sp. PO with 2.1 x 1.4 µm (length and width), the spore size of the *Nosema* sp. Phop was much larger with 3.6 x 2.0 µm in average. The number of polar filament coils is one criterion used in discriminating *Nosema* species (Burges et al., 1974).

In conclusion, a potentially new microsporidium isolated from *P. operculella* was purified and characterized. Its morphological characteristics, including spore size, polar filament coils and different stages of the life cycle as well as its genetic relationship to other *Nosema/Vairimorpha* species indicated that this microsporidium belongs to a previously undescribed *Nosema* species. This microsporidium infected the fat body and midgut epithelium of larvae of *P. operculella* and apparently affected larval susceptibility to PhopGV-GR1.1, resulting in an antagonistic interaction, visible as the loss of the host´s response to virus challenge. Infection experiments are often designed to analyse the effect of one pathogen to its host. Our study, however, indicates that mixed infections with different pathogens may significantly influence bioassay data, which would result in erroneous determinations of virulence parameters, such as LC_{50}, if such mixed infections were not recognized. Therefore, a careful diagnostic survey of host insect populations is an essential prerequisite for any virulence test.

Chapter VI

General Discussion

This thesis was adressed to investigate the diversity of PhopGV by the characterization of different isolates derived from different geographic origins. This aim was achieved by testing the biological activity of PhopGV isolates against *P. operculella* larvae (Chapter II), the full genome sequencing and analysis of 12 isolates (Chapter III), as well as studying the interaction of peroral infectivity in the presence of an internal virus (Chapter IV) and a microsporidium infection (Chapter V).

It was demonstrated that PhopGV isolates show a different biological activity expressed as LC_{50} and LT_{50}, but virulence of a single isolate was host dependend as well. This was shown by infecting two different *P. operculella* populations (Phop-IT and Phop-TN) with the same PhopGV concentration, resulting in different mortality values. The analysed PhopGV isolates seemed to show a wide difference in the biological activity against the *P. operculella* population Phop-IT at 14 days post infection. The LC_{50} values were such apart that some isolates appeared as highly virulent, while others caused no apparent mortality. But PhopGV is a slow-killing virus (Gómez Valderrama et al., 2017) which showed a low mortality on day 14 and was effective at a later point of time, after day 21. Hence, also the less active isolates which did not cause a mortality against *P. operculella* after 14 dpi may offer a sufficient efficacy against their host on a later point of time, but for this hypothesis more biotest data is needed, evaluated 21 dpi. Infected larvae were retarded in development and failed pupation as described by Sporleder et al. (2005). The individuals which stuck in the larval stage finally died in this stage. As a result, the following host generation may become massively reduced, caused by the incident that more than 90% of the *P. operculella* population did not reach the adult stage as was therefore unable to produce eggs to build up the next generation. This provides the approach to control whole insect populations with a long-term strategy instead of a short-term strategy of fast-killing pest insects after acute high infestations. Fast-killing baculoviruses (Federici, 1997) have the potential to control pests more efficiently in the short-term than slow-killing ones, but slow-killing viruses may have a higher vertical transmission efficiency and may control pests effectively in the long-term (Takahashi et al., 2015). Nevertheless, single isolates (PhopGV-GR1) showed a higher killing speed compared to other isolates. But this effect was specific for the Phop-IT population and could not be directly transferred to other insect populations, like Phop-TN. Thus, the biological activity of PhopGV, with the parameters LC_{50} and LT_{50}, not only seems to be isolate but also host strain dependent, even when different *P. operculella* populations were reported to show a rather similar reaction against the same PhopGV isolates (Zeddam et al., 2013, Gómez-Bonilla et al., 2011a). Differences in virus activity were previously described if the same isolate was used to infect different *Gelechiid* species. For example the relative potency of PhopGV towards *P. operculella* and *T. solanivora* larvae highly depended on the isolates considered (Zeddam et al., 2013). The finding, that PhopGV is able to infect different host species however showed different virulence against different host species was explained by adaptation to host species, after having previously contact with only one host or contact with more than one host (Gómez-Bonilla et al., 2011a; Gómez-Bonilla et al., 2011b; Espinel-Correal et al., 2010). The adaptation to different populations of the same host species or to a number of different host species may be one reason that PhopGV often occurs in mixtures of genotypic variants, well adapted for replication in different hosts (Gómez-Bonilla et al., 2011b). Mixtures of genotypic variants were previously detected for PhopGV isolates by RFLP and analysis of the *egt* types (Gómez-Bonilla et al., 2011b). This multiple genetic variants of single virus isolates could be confirmed by whole genome sequencing for the majority of the sequenced PhopGV isolates in this study. Further, the high potential of the analysed PhopGV isolates to go into mixture with PhopGV-R after a passage in Phop-IT is a hint that PhopGV often tolerates genotype mixtures. The

high number of isolates which were showing mixtures of different genetic variants indicates that this strategy provides a number of advantages like the following described examples affirm. A significantly higher virulence was observed in both *P. operculella* and *T. solanivora* when PhopGV isolates corresponding to a natural mixture of viral genotypes were used (Espinel-Correal et al., 2010). Besides the potential to infect different host species more easily because a mixture of genetic variants is more universal than one genotype which is highly specific, the second benefit could be the increased potential to overcome adaptations of the host to avoid a resistance development. It was previously demonstrated that one way of building resistance of *P. operculella* against PhopGV is controlled by classical Mendelian factors (Briese, 1982). Further, PhopGV occurs worldwide together with the distribution of its various hosts (Espinel-Correal et al., 2010; Zeddam et al., 1999; Kroschel and Koch, 1996; Hunter et al., 1975). The different climatic zones may cause adaptations of the host to the changed environmental conditions, and as a consequence multiple virus genotype variants can then be a source for selection for genotypes which fit best to the changed conditions. Interestingly, the multiple genetic variations did not affect the genome stability. All analysed isolates showed 130 ORFs and no additional ORF was detected. But many small changes visible as SNPs were detected along the PhopGV genomes of the different isolates. Every analysed isolate showed isolate-specific SNPs which were not present in any of the other analysed isolates. But the number of total SNPs per genome was relatively low compared to other betabaculoviruses, *e.g.* CpGV. The frequencies of the detected SNPs indicated the presence of multiple genotype variants but the number of different variants was too high to identify or discriminate a single genotype from others. Only a consensus of the majority variants could be built. But a grouping on the isolate level was possible considering SNPs and Indel information. Further, a variability of the gene *sod* (ORF 54) was detected in this study. The *sod* length variants on the coding sequence level can serve as additional tool for variant identification and grouping of PhopGV isolates besides the already known grouping based on *egt* gene polymorphisms. The internal virus isolate PhopGV-R for example shows one *egt* type but two different *sod* types. These two marker genes can answer the question if isolates consist of genetic variant mixtures and further allow their quantification. PhopGV isolates collected from Italy and Greece appeared nearly pure; each isolate showed only one *egt* type and also only one *sod* type. This result was confirmed analysing SNP frequencies, with values close to 1 which indicated high genetic homogeneity. For isolate PhopGV-CR3.1 the SNP frequency values were fluctuating, this indicated a mixture of at least two genotype variants. The exact number of variants could not be determined by analysing the SNP frequency values, but the genotype variants could be assigned to two different *egt* and two *sod* types and a quantification of these different types was possible. In summary, PhopGV isolates can be assigned and sorted after group specific SNPs and Indels. Marker genes like *egt* and *sod* allow a quantification of genetic variant types on the single isolates level.

Persistent covert baculovirus infections have been detected in lepidopteran insect populations in the laboratory and also in the field (Vilaplana et al., 2010; Burden et al., 2003). There are theories that persistent infections cannot be cured and are likely the result of surviving virus challenge (Vilaplana et al., 2010; Cory and Myers, 2003; Andrealis, 1987). It has been demonstrated that sublethal infections with betabaculoviruses like Plodia interpunctella granulovirus (PiGV) introduced a persistent infection in a *Plodia interpunctella* population (Burden et al., 2002). In many insect populations *e.g. Spodoptera exigua* and *Mamestra brassicae* sublethal infections with an alphabaculovirus (SeMNPV, MabrMNPV) led to a vertical virus transmission from adults to offspring (Burden *et al.*, 2003; Kukan, 1999; Goulson and Cory, 1995; Smith and Vlak, 1988).

It seems to be a long-term survival strategy of the virus to adapt to or manipulate the life cycle of the host (Moore, 2002), in contrast to highly virulent parasites and pathogens which will following ecological models become extinct ('fade-out') because of a rapidly exhaustion of the pool of

susceptible hosts (Wobeser, 2006; Earn et al., 1998, Swinton et al., 1998; Keeling and Grenfell, 1997). Further, naturally outbreaks of overt baculovirus infections are rare (Myers, 1988) and horizontal virus transmission is inefficient at low host densities (Anderson and May, 1981). The latter is not only true because of the reduced probability for host ingestion at low host population densities, but also influenced by UV radiation which can reduce infectivity of the virus 10 to 100-fold within days (Jones et al., 1993; Biever and Hostetter, 1985). When considering the chance of higher distribution rates, better survival chances at low-host densities and the prevention of damage by UV light, a selection for low virulence may lead to persistent, vertically transmitted covert baculovirus infections.

In all three analysed *P. operculella* populations Phop-IT, Phop-EG and Phop-TN, the occurrence of a persistent covert PhopGV infection was demonstrated. Together with evidence from other baculovirus-host systems, as mentioned above, it is likely that insect populations in the laboratory and the field carry persistent covert baculovirus infections. Sublethal infections with betabaculoviruses may introduce a persistent virus infection into an insect population. It can be assumed that insect populations likely carry a potential virus infection and a totally virus free insect population is not the norm but an exception.

Another question is whether insect populations which survived a sublethal virus infection can be cured and produce virus free offspring again or if inevitably an establishment of persistent covert infection takes place. Resistance building of the host *P. operculella* against some genetic PhopGV variants was reported (Sporleder, 2003; Briese, 1982). But in the case of persistent sublethal infections it is unlikely that a resistace building of Phop populations against PhopGV will happen because no selection can happen under virus pressure.

This study showed that a persistent covert infection can become overt. A crowding effect was described in the literature for being a stressor for covert infected insect colonies to start an active overt infection in *Trichoplusia ni* populations (Fuxa, 1999), *Colias philodice eurytheme* and *Junonia coenia* (Steinhaus, 1958), *Mamestra brassica* (Burden et al., 2006; Hughes et al., 1993) and *Thaumatotibia leucotreta* (Opoku-Debrah et al., 2013) but this effect was not observed for *P. operculella* in this study. Only a weak evidence for correlation of larval density and virus outbreak was present, but this effect was not statistically significant. Further, the activation of covert infections to overt infections by infection with a second virus were reported. The second virus can be either homologous or even heterologous like another baculovirus species (Murillo et al., 2011; Burden et al., 2003; Cooper et al., 2003; Hughes et al., 1997 and 1993; Kelly et al., 1981). This triggering effect was also observed for PhopGV in this study when individuals of *P. operculella* were infected by certain PhopGV isolates, for example with PhopGV-CR3, originating from Costa Rica. Interestingly, other Mediterranean isolates from Italy or Greece suppressed the internal virus. If larvae were infected with such an isolate, the internal covert persistent virus could not be detected anymore. Even when the purified internal virus was applied together with a PhopGV-GR1.1 as a co-infection of single *P. operculella* neonate larvae, no internal virus was detected but only the PhopGV-GR1.1. There was either a selection for the higher virulent PhopGV isolate, which would be supported by the biological activity tests, where isolates which suppress the internal virus showed a lower LC_{50} compared to isolates which go into mixture with the internal virus, or a not yet determined blocking mechanism. It has been reported that baculoviruses exclude secondary infection, a so called “superinfection exclusion” by a rearrangement of the actin filaments, preventing the transportation of a second virus to the host cell nucleus (Beperet et al., 2014). If such a mechanism also exists for *P. operculella* cells and PhopGV could not be tested because there is no cell culture for *P. operculella* available.

Interestingly, the *P. operculella* colony Phop-IT could be perorally infected by its own internal virus PhopGV-R, that had been purified from spontaneously diseased larvae and was re-applied to the diet of neonate larvae. In the described case the peroral infection was overt and the larvae died due to infection with this virus. Like for other tested PhopGV isolates there was also a reduction in the pupation rate observed, which was virus concentration depended.

PhopGV not only showed interactions with other PhopGV isolates but also interactions with other pathogens, for instance in co-infections of single larvae which carried the microsporidium *Nosema* sp. Compared to microsporidia-free larvae, the co-infected larvae were smaller in size, showed weak lethargic behaviour but did not die as expected after a successful PhopGV infection. The presence of the microsporidium caused a considerable shift towards higher PhopGV concentrations needed to achieve the same LC_{50} against *P. operculella* larvae after 14 dpi, compared to neonate larvae infected with PhopGV alone. This effect was most likely driven by microsporidian activity in infected host cells. Reduced larval size was reported after infection of neonate and third instar *Agrotis ipsilon* larvae with *Vairimorpha* sp. and *Vairimorpha necatrix* that also led to less tissue mass for virus infection with Rachiplusia ou multiple nucleopolyhedrovirus (RoMNPV) if a double infection took place (Cossentine and Lewis, 1984). Inhibiting apoptosis appears to be a common mechanism used by microsporidia (Del et al., 2006; Scanlon et al., 1999). For *Bombyx mori* cells, it has been reported that *N. bombycis* prevents apoptosis and reactive oxygen species (ROS) production to create the optimal environmental condition for its growth and development (He et al., 2015). Perhaps the microsporidium also attenuates the negative effects of a baculovirus infection to the host cell. Virus activity would destroy the infected cells, which would lead to death of the host and the release of OB in consequence earlier without the influence of the microsporidium. The host may die on a later stage at low virus concentrations, to allow the microsporidium to complete its life cycle and reproduce before death of the host. This may help to keep microsporidia infection in the population of *P. operculella*. Many host-pathogen systems indicate that “concomitant” or “mixed” infections involving two or more parasite species or genotypes are common and may even be the rule (Thomas et al., 2003; Cox, 2001; Read and Taylor, 2001). In the case of the identified *Nosema* sp. morphologically and genetically belonging to the “true *Nosema*” clade an antagonistic interaction against PhopGV in *P. operculella* larvae was observed.

From the study presented here, important elements can be learned for improving plant protection strategies. Low amount of virus or low virulent isolates can help to establish long-term effects on insect populations or higher amounts and more virulent isolates can kill a high number of individuals of a population short-term. Further mixtures of variant genotypes occurring in the field and can be generated in the laboratory. If PhopGV in particular and baculoviruses in general are applied as active substance against *P. operculella* it is important to consider if and how the isolate interacts with other natural occurring isolates and if the applied isolate goes into mixture or will supress other genotype variants. Such interactions may explain different susceptibilities of different *P. operculella* target populations. Also other pathogen species, such as microsporidia, need to be considered because their presence can alter the virus caused mortality against the host. For future research a cell culture and virus expression system for PhopGV is strongly needed to verify if observed differences in the genome cause differences in the virus function.

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Curriculum Vitae

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Academic Studies	02/2014- 11/2018	PhD student at the Julius Kühn-Institut, Federal Institute for Biological Control Darmstadt, Germany EU founded project: BIOCOMES PhopGV baculoviruses for control of *Tuta absoluta* in tomato and *Phthorimaea operculella* and *Tecia solanivora* in potato
	2013	Master Thesis with the topic: “Characterization of snake venom impact on heterologous expressed ligand-gated ion channels (LGICs) via two-electrode voltage-clamp (TEVC)”
	2012	Visiting Fellow at the Australian National University (ANU), Plant Science Division, Research School of Biology in Canberra Practical and Compulsory Courses to Rising CO_2, Photosynthesis and Plant Growth
	2011 - 2013	Course of studies Biology (Master of Science) on the Technical University Darmstadt
	2011	Bachelor Thesis with the topic: “Characterization of Rice aquaporin RNAi lines”
	2008 - 2011	Course of studies Biology (Bachelor) on the Technical University Darmstadt

Publications

Larem A., Ben-Tiba S., Wennmann J.T. and Jehle J.A. (2018). Effects of a covert infection with an internal Phthorimaea operculella granulovirus in insect populations of Phthorimaea operculella. Viruses, Submitted

Larem A., Wennmann J.T., Ben-Tiba S., Gueli-Alletti G. and Jehle J.A. (2018). Elucidating the genetic diversity of Phthorimaea operculella granulovirus (PhopGV). JGV, Submitted

Larem A., Fritsch E., Undorf-Spahn K., Kleespies R.G. and Jehle J.A. (2018). Interaction of Phthorimaea operculella granulovirus with a microsporidium (Nosema sp. Phop) in larvae of Phthorimaea operculella.JIP, Submitted.

Ben Tiba S., Larem A., Laarif A., Fritsch E., Undorf-Spahn K., Abuelgasim Mohamed S., Ekesi S., Wennmann J.T., Kroschel J., Fattouch S., Jehle J.A. (2018). The potential of novel African isolates of Phthorimaea operculella granulovirus for the control of Tuta absoluta. Journal of Applied Entomology.

Jehle J.A., Herz A, Keller B., Kleespies R.G., Koch E., Larem A., Schmitt A., Stephan D. (2013). Status Report Biological Plant Control 2013, JKI Publication: 173

Abstracts

Larem A., Fritsch E., Undorf-Spahn K. and Jehle J. A. (2016). Identifizierung neuer Isolate des Phthorimaea operculella Granulovirus (PhopGV) zur kombinierten Bekämpfung von Phthorimaea, Tuta und Tecia. 60. Deutsche Pflanzenschutztagung, Halle, Germany.

Larem A., Fritsch E., Undorf-Spahn K., Wennmann J.T. and Jehle J.A. (2016). Whole Genome Sequencing of PhopGV Isolates for Control of Tuta absoluta in Tomato and Phthorimaea operculella and Tecia solanivora in Potato. 49th Annual Meeting of the Society for Invertebrate Pathology and International Congress on Invertebrate Pathology and Microbial Control, Tours, France.

Larem A., Fritsch E., Undorf-Spahn K. and Jehle J.A. (2015). PhopGV for control of Tuta absoluta in tomato and Phthorimaea operculella and Tecia solanivora in potato. 48th Annual Meeting of the Society for Invertebrate Pathology and International Congress on Invertebrate Pathology and Microbial Control, Vancouver, Canada.

Jehle, J.A.; Herz, A.; Keller, B.; Kleespies, R.G.; Koch, E.; Larem, A.; Schmitt, A.; Stephan, D. (2014). The Use of Microbial Plant Protection Agents for Insect Control in Germany. 47th Annual Meeting of the Society for Invertebrate Pathology and International Congress on Invertebrate Pathology and Microbial Control, Mainz, Germany

Larem A., Fritsch, E., Undorf-Spahn, K. and Jehle, J.A. (2014). PhopGV baculoviruses for control of *Tuta absoluta* in tomato and *Phthorimaea operculella* and *Tuta solanivor*a in potato. 59. Deutsche Pflanzenschutztagung, Freiburg, Germany.

Larem A., Fritsch, E., Undorf-Spahn, K. and Jehle, J.A. (2014). PhopGV baculoviruses for control of T. absoluta in tomato and P. operculella and T. solanivora in potato. 7th Young Scientists Meeting, Quedlinburg, Germany.

Honors and Awards

Price for the best oral presentation at the 7th Young Scientists Meeting (2014), Quedlinburg, Germany.

Supplementary

Table S III 1. Position and orientation of 130 putative ORFs predicted for PhopGV isolates. The ORFs of the different isolates differ to the reference isolate PhopGV-1346 in terms of length or amino acid sequence. The differences are highlighted in bold letters, aa length which differ from the reference and the refering ORFs are underlined. The reading direction is indicated forward (>) and reverse (<).

Table S III 2. All SNP positions and appropriate frequencies of the sequenced PhopGV isolates in relation to the reference isolate PhopGV-1346. Phred-scaled probability of all samples being homozygous to reference (QUAL); read depth (DP); number of reads covering reference forward, ref. reverse, alternative for. and alt. rev. (DP4); read depth of different alleles for reference, alternative 1, alt. 2 and alt. 3 (DPR) and genotype quality value (GQ) are indicated.

For Supplementary Tables S III 1 and S III 2 see attached file.

Table S III 3. List of group specific SNPs and frequencies in relation to the reference isolate PhopGV-1346. Change of reference to alternative base (REF -> ALT); read depth (DP) and alternative frequency (FREQ ALT) are indicated.

Group 1		LS1.1		LS1.2		LS3.1		CR3.1		R	
POSITION	REF->ALT	DP	FREQ ALT	DP	FREQ ALT	DP	FREQ ALT	DP	FREQ ALT	DP	FREQ ALT
7882	T->A	608	29.1	565	39.6	517	41.4	520	22.5	583	79.6
11291	T->C	548	65.3	556	68.5	498	34.7	504	26.2	572	89.0
21377	C->A	616	31.2	570	34.2	523	18.0	485	37.5	586	82.9
34136	G->T	546	21.2	547	30.7	468	23.5	484	27.7	546	7.5
35372	G->T	650	41.2	756	36.1	514	45.5	514	28.8	727	73.6
50800	C->A	577	21.8	544	24.6	496	34.3	488	41.6	565	86.9
85232	A->C	542	49.3	522	54.8	464	28.9	466	32.6	564	92.0
115269	C->T	583	36.4	613	33.0	502	39.6	502	30.1	583	99.7

Group 2		YM.1		LS2.1	
POSITION	REF->ALT	DP	FREQ ALT	DP	FREQ ALT
974	G->T	504	86.7	669	98.2
4723	C->T	504	88.7	614	24.3
5408	C->T	483	89.2	595	23.2
9561	C->T	493	38.1	689	22.4
9622	G->A	476	46.4	562	72.8
9921	C->T	484	46.5	613	77.7
10940	G->A	580	50.9	780	76.2
11023	G->T	581	42.0	735	23.4
13382	G->A	502	89.4	599	99.0
14640	C->T	507	89.0	601	22.5
17756	G->T	388	72.9	433	98.2
21233	T->G	470	88.9	653	98.9
23712	T->C	476	93.1	658	99.5
27489	C->T	512	42.2	700	27.9
33956	C->T	441	84.4	608	17.9
36586	A->G	528	89.0	671	22.4
37431	A->T	498	38.2	724	80.8
41481	A->G	359	33.1	450	44.4
41484	G->C	350	33.7	434	46.1
41486	A->C	350	33.7	434	46.1
41495	A->G	360	30.6	442	33.9
48088	C->T	457	86.0	610	19.5
48351	G->A	493	90.5	543	23.0
55180	A->T	465	84.5	573	100.0
55357	A->C	444	47.7	520	85.4
56349	T->C	500	47.8	575	78.8
67563	C->T	481	79.4	545	80.0
76726	C->A	477	75.7	571	93.2
81581	A->T	429	25.2	568	31.0
87209	G->T	455	76.7	530	81.5
92351	C->A	428	50.2	595	20.5
92872	A->G	422	24.9	553	86.4
97276	G->A	657	87.7	936	99.8
101759	G->A	547	58.7	595	92.1
103061	C->T	528	64.2	620	81.5
103062	C->T	529	64.1	623	80.6
105647	T->C	493	79.5	616	92.2
106115	T->C	482	78.8	625	92.3
107039	G->A	507	88.4	626	99.2
112811	C->T	440	86.1	618	99.4
114951	C->T	462	59.5	644	92.2
116937	C->T	516	89.1	619	99.7

Group 3		IT1.1		GR1.1		
POSITION	REF->ALT	DP	FREQ ALT	DP	FREQ ALT	DP
1762	T->C	596	93.8	494	98.8	594
2627	T->C	604	99.3	474	97.0	588
2660	C->T	574	99.1	431	96.1	562
7100	T->A	520	99.4	477	70.9	494
7157	T->C	541	95.0	532	71.1	539
7219	C->T	535	99.4	511	99.2	617
7242	T->C	498	98.2	487	69.6	576
7344	A->T	493	98.2	504	69.0	571
7357	T->C	524	98.1	495	67.5	585
7490	A->G	543	99.3	529	98.7	663
7565	A->G	526	98.3	522	71.3	565
7674	A>G	541	98.7	543	90.8	580
7724	A->G	579	97.6	536	95.1	647
7804	A->T	634	98.3	576	73.4	696
12879	A->G	239	97.1	208	93.3	230
13314	C->T	294	95.9	173	92.5	275
21801	G->A	588	93.7	502	93.6	603
23799	T->G	532	98.7	485	74.8	553
23803	G->C	588	99.0	507	74.6	603
23820	A->C	496	99.0	507	72.4	563
23845	A->G	562	98.8	511	71.6	584
25271	A->T	563	91.8	465	71.8	547
26207	T->C	516	98.3	383	39.9	514
41824	T->C	523	98.1	383	99.2	591
44346	C->T	538	99.8	490	98.0	498
44385	G->A	479	99.0	426	97.9	504
60491	A->T	534	99.1	443	18.7	496
61861	C->T	565	98.9	481	98.3	574
89586	C->A	565	99.5	496	98.2	602
99734	A->G	588	99.1	528	98.3	606
107112	G->T	587	99.8	485	99.2	609
110566	T->C	573	94.2	442	24.7	615

Group 4		CR5.1	
POSITION	REF->ALT	DP	FREQ ALT
24018	G->A	539	70.9
39994	G->A	495	43.8
40027	C->A	477	23.7
53963	G->T	370	43.5
53980	G->A	452	38.5
83936	G->T	475	58.9

Table S III 4. List of isolate unique SNPs and frequencies. SNP positions are relative to the reference isolate PhopGV-1346. Change of reference to alternative base (REF -> ALT); read depth (DP) and alternative frequency (FREQ ALT) are indicated. The isolates are sorted after the group (1-4) they are assigned to. The numbers in brackets representing the total number of isolate unique SNPs.

Group 1

LS1.1 (13)					LS1.2 (11)				
POSITION	REF->ALT	DP	ALT	FREQ ALT	POSITION	REF->ALT	DP	ALT	FREQ ALT
12844	G->A	566	307	0.5	12844	G->A	534	228	0.4
29046	T->C	610	136	0.2	29046	T->C	540	123	0.2
31514	G->T	106	17	0.2	29515	G->C	570	174	0.3
31515	T->G	107	18	0.2	31514	G->T	204	45	0.2
31537	T->C	57	11	0.2	31515	T->G	206	47	0.2
31562	T->G	71	15	0.2	40934	T->C	686	164	0.2
33414	G->T	557	173	0.3	44677	T->C	567	228	0.4
44677	T->C	553	282	0.5	50931	T->C	566	124	0.2
50931	T->C	597	146	0.2	77156	G->A	524	83	0.2
67692	G->T	591	142	0.2	89730	G->A	579	133	0.2
89730	G->A	604	123	0.2	91220	A->G	530	238	0.4
91220	A->G	574	196	0.3					
98986	A->G	557	146	0.3					

LS3.1 (24)					CR3.1 (24)				
POSITION	REF->ALT	DP	ALT	FREQ ALT	POSITION	REF->ALT	DP	ALT	FREQ ALT
6859	C->A	493	137	0.3	7371	G->A	521	95	0.2
7971	G->A	515	200	0.4	10040	A->C	482	93	0.2
13143	A->G	467	133	0.3	16144	T->A	476	129	0.3
20090	G->T	487	148	0.3	19364	A->G	491	122	0.2
45515	C->T	513	191	0.4	19808	C->T	478	80	0.2
47525	C->T	508	154	0.3	20306	C->T	520	111	0.2
48099	C->A	486	153	0.3	21645	C->T	504	132	0.3
48311	A->T	489	136	0.3	27382	T->C	493	151	0.3
55649	C->T	501	159	0.3	29237	G->A	485	133	0.3
56932	A->G	495	105	0.2	45394	G->A	494	196	0.4
57380	C->T	650	168	0.3	51485	T->G	498	85	0.2
63894	C->T	521	198	0.4	56420	A->G	523	114	0.2

64769	C->T	548	159	0.3	65422	C->T	519	120	0.2
75940	G->A	486	180	0.4	71852	A->G	471	138	0.3
79610	G->A	462	117	0.3	76364	T->C	505	114	0.2
81662	C->T	477	123	0.3	78462	G->A	492	97	0.2
88051	T->C	503	150	0.3	78683	C->A	461	90	0.2
93605	C->T	501	197	0.4	86986	C->T	506	103	0.2
110608	G->A	491	128	0.3	92104	G->A	517	97	0.2
110653	T->C	486	132	0.3	97555	T->C	479	138	0.3
113361	T->C	492	94	0.2	105999	G->T	493	91	0.2
113418	T->C	470	151	0.3	110818	A->G	487	120	0.2
115516	G->A	501	118	0.2	113803	T->A	481	93	0.2
115752	C->T	512	156	0.3	115326	C->T	508	111	0.2

Group 2

YM1.1 (28)					LS2.1 (12)				
POSITION	**REF->ALT**	**DP**	**ALT**	**FREQ ALT**	**POSITION**	**REF->ALT**	**DP**	**ALT**	**FREQ ALT**
723	T->G	495	264	0.5	5056	C->T	619	479	0.8
15831	C->T	483	159	0.3	31581	C->T	359	171	0.5
19893	G->A	487	214	0.4	32236	G->A	628	462	0.7
23048	A->T	249	170	0.7	56984	C->T	701	617	0.9
28988	G->A	516	194	0.4	59750	C->A	591	424	0.7
30623	T->G	558	311	0.6	78917	C->T	657	492	0.7
31412	G->T	307	79	0.3	94705	T->G	605	441	0.7
31413	T->G	295	75	0.3	95331	T->G	402	130	0.3
37358	A->G	480	243	0.5	97078	C->T	618	578	0.9
39317	G->A	344	154	0.4	103931	C->T	596	455	0.8
39320	T->C	453	159	0.4	115630	A->C	315	162	0.5
42612	G->A	485	196	0.4	115631	G->A	316	169	0.5
45764	A->G	533	243	0.5					
54082	C->T	488	407	0.8					
55341	G->A	481	174	0.4					
57733	C->T	499	435	0.9					
61231	A->T	512	306	0.6					
73695	A->G	493	235	0.5					
76389	G->A	505	397	0.8					
81599	T->A	366	97	0.3					
90143	C->T	475	254	0.5					
92348	T->C	423	203	0.5					
96361	T->A	469	223	0.5					
99148	C->T	517	396	0.8					
99957	C->T	497	360	0.7					
99958	C->T	493	360	0.7					
101108	G->A	518	367	0.7					
104407	C->T	496	230	0.5					

Group 3

GR1.1 (4)					GR1.2 (4)				
POSITION	**REF->ALT**	**DP**	**ALT**	**FREQ ALT**	**POSITION**	**REF->ALT**	**DP**	**ALT**	**FREQ ALT**
26498	C->T	482	66	0.1	26498	C->T	601	112	0.2
26555	A->G	482	71	0.1	26555	A->G	562	111	0.2
38281	C->T	486	254	0.5	38281	C->T	625	345	0.6
46199	T->G	543	383	0.7	46199	T->G	754	457	0.6
52509	G->A	455	112	0.2	52509	G->A	617	58	0.1

GR2.1 (12)					IT1.1 (1)				
POSITION	**REF->ALT**	**DP**	**ALT**	**FREQ ALT**	**POSITION**	**REF->ALT**	**DP**	**ALT**	**FREQ ALT**
16249	A->C	657	236	0.4	117126	C->T	556	549	1.0
40036	A->G	662	329	0.5					
52830	T->C	596	201	0.3					
60732	C->T	600	178	0.3					
67679	G->A	432	110	0.3					
67682	G->C	432	107	0.2					
67686	G->A	492	106	0.2					
67713	G->A	606	326	0.5					
67968	A->T	712	480	0.7					
92682	G->A	614	213	0.3					
96571	A->G	608	474	0.8					
96646	A->G	620	538	0.9					

Group 4

CR5.1 (6)				
POSITION	**REF->ALT**	**DP**	**ALT**	**FREQ ALT**
24018	G->A	539	382	0.7
39994	G->A	495	217	0.4
40027	C->A	477	113	0.2
53963	G->T	370	161	0.4
53980	G->A	452	174	0.4
83936	G->T	475	280	0.6

Table S IV 1. Annotation of PhopGV-R compared to the reference isolate PhopGV-1346. Differences in the amino acid length are highlighted with bold red numbers. The dedicated reference was highlighted with bold blue numbers.

		PhopGV-1346			PhopGV-R		
ORF	Name	Position	length (bp)	length (aa)	Position	length (bp)	length (aa)
1	*granulin*	1 > 747	747	248	1 > 747	747	248
2	*P78/83*	883 < 1404	522	173	883 < 1404	522	173
3	*pk-1*	1385 > 2221	837	278	1385 > 2221	837	278
4		2357 < 2929	573	190	2386 < 2958	573	190
5		2922 > 3161	240	79	2951 > 3190	240	79
6	*ie-1*	3538 < 4818	1281	426	3567 < 4847	1281	426
7		4842 > 5459	618	205	4871 > 5488	618	205
8		5479 < 5850	372	123	5508 < 5879	372	123
9	*bro*	6003 > 7079	1077	358	6064 > 7140	1077	358
10		7181 > 7351	171	56	7242 > 7412	171	56
11		7638 > 7877	240	79	7699 > 7938	240	79
12	*ODV-e18*	8027 < 8278	252	83	8088 < 8339	252	83
13	*P49*	8279 < 9559	1281	426	8340 < 9620	1281	426
14		9769 < 10386	618	205	9831 < 10448	618	205
15		10475 < 11434	960	319	10529 < 11488	960	319
16	*ODV-e56*	11464 < 12519	1056	351	11518 < 12573	1056	351
17		12634 > 12822	189	62	12688 > 12876	189	62
18		13124 < 13285	162	53	13178 < 13339	162	53
19		13354 < 13827	474	157	13408 < 13881	474	157
20		13890 > 14798	909	302	13944 > 14852	909	302
21		14824 > 15264	441	146	14878 > 15318	441	146
22		15315 > 15680	366	121	15369 > 15734	366	121
23		15754 > 16068	315	104	15808 > 16122	315	104
24	*PE-38*	16369 < 17508	1140	**379**	16423 < 17559	1137	**378**
25		18497 > 19639	1143	380	18546 > 19688	1143	380
26		20524 > 21063	540	179	20573 > 21112	540	179
27	*efp*	21193 > 22980	1788	595	21242 > 23029	1788	595
28		23479 > 25089	1611	**536**	23528 > 25123	1596	**531**
29		25295 < 25957	663	220	25293 < 25955	663	220
30		26339 < 26902	564	187	26336 < 26899	564	187
31		26906 > 27439	534	177	26903 > 27436	534	177
32		27436 < 29463	2028	675	27433 < 29460	2028	675
33	*ODV-e66*	29470 < 31860	2391	**796**	29467 < 31833	2367	**788**
34		31911 > 32225	315	104	31884 > 32198	315	104
35		32360 < 32653	294	97	32333 < 32626	294	97
36		32826 > 33161	336	111	32826 > 33161	336	111
37	*lef-2*	33154 > 33696	543	180	33154 > 33696	543	180
38		33951 > 34208	258	85	33951 > 34208	258	85
39		34229 < 34573	345	114	34229 < 34573	345	114
40		34720 < 35073	354	117	34720 < 35073	354	117

41	*mp-nase*	35132 < 36541	1410	469	35132 < 36541	1410	469
42	*p13*	36546 > 37379	834	277	36546 > 37379	834	277
43		37402 > 37716	315	**104**	37402 > 37710	309	**102**
44		37732 > 38832	1101	366	37726 > 38826	1101	366
45		39075 < 39278	204	67	39070 < 39273	204	67
46		39303 > 41735	2433	810	39298 > 41730	2433	810
47		41668 < 42576	909	302	41663 < 42571	909	302
48		42588 > 42731	144	47	42583 > 42726	144	47
49	*v-ubi*	42825 < 43109	285	94	42820 < 43104	285	94
50		43229 > 44248	1020	339	43224 > 44243	1020	339
51		44260 > 44406	147	48	44255 > 44401	147	48
52	*PP31*	44421 < 45116	696	231	44416 < 45111	696	231
53	*lef-11*	45100 < 45366	267	88	45095 < 45361	267	88
54	*sod*	45531 < 45850	390	129	45446 < 45835	390	129
55	*p74*	46284 < 48260	1977	658	46270 < 48246	1977	658
56		48436 < 48852	417	138	48422 < 48838	417	138
57		49042 > 49458	417	138	49028 > 49444	417	138
58		49702 > 50475	774	257	49688 > 50461	774	257
59		50673 < 51266	594	197	50659 < 51252	594	197
60		51354 < 51872	519	172	51340 < 51858	519	172
61	*p47*	51966 > 53165	1200	399	51952 > 53151	1200	399
62		53247 > 53894	648	215	53233 > 53880	648	215
63	*p24*	54103 > 54606	504	167	54089 > 54592	504	167
64		54608 > 55165	558	185	54594 > 55151	558	185
65	*p38.7*	55443 < 56000	558	185	55430 < 55987	558	185
66	*lef-11*	55894 < 56595	702	233	55881 < 56582	702	233
67		56788 > 58356	1569	522	56775 > 58343	1569	522
68		58379 > 58543	165	54	58366 > 58530	165	54
69	*fgf-1*	58591 < 59310	720	239	58578 < 59297	720	239
70		59452 < 59769	318	105	59439 < 59756	318	105
71		60034 > 60492	459	152	60021 > 60479	459	152
72	*lef-6*	60579 < 60863	285	94	60566 < 60850	285	94
73	*dbp*	61039 < 61872	834	277	61026 < 61859	834	277
74		62051 < 62578	528	175	62038 < 62565	528	175
75		62730 > 63869	1140	379	62717 > 63856	1140	379
76		64031 > 64357	327	108	64032 > 64358	327	108
77	*BV/ODV-c42*	64439 > 65578	1140	379	64440 > 65579	1140	379
78	*p6.9*	65595 > 65759	165	54	65596 > 65760	165	54
79	*lef-5*	65908 < 66630	723	240	65910 < 66632	723	240
80		66583 > 67482	900	299	66585 > 67484	900	299
81		67991 < 68479	489	162	67993 < 68481	489	162
82	*helicase 1*	68463 > 71864	3402	1133	68465 > 71866	3402	1133
83	*ODV-e25*	72037 < 72681	645	214	72039 < 72683	645	214
84		72812 < 73297	486	161	72814 < 73299	486	161
85		73324 > 74082	759	252	73326 > 74084	759	252
86	*iap-Op1*	74312 < 75049	738	245	74313 < 75050	738	245

87	*lef-4*	75075 < 76400	1326	441	75076 < 76401	1326	441
88	*vp39*	76455 > 77336	882	293	76456 > 77337	882	293
89	*ODV-ec27*	77442 > 78305	864	287	77443 > 78306	864	287
90		78469 > 78621	153	50	78470 > 78622	153	50
91		78629 > 78784	156	51	78630 > 78785	156	51
92		78733 < 79944	1212	403	78734 < 79945	1212	403
93		79931 > 80224	294	97	79932 > 80225	294	97
94	*vp91*	80297 < 82435	2139	**712**	80298 < 82418	2121	**706**
95	*tlp20*	82410 > 82763	354	117	82393 > 82746	354	117
96		82747 > 83418	672	223	82730 > 83401	672	223
97	*gp41*	83300 > 84157	858	285	83283 > 84140	858	285
98		84423 > 84680	258	85	84406 > 84663	258	85
99	*vlf-1*	84637 > 85782	1146	381	84620 > 85765	1146	381
100		85976 > 86233	258	85	85959 > 86216	258	85
101		86281 > 86718	438	145	86264 > 86701	438	145
102		86912 < 87277	366	121	86895 < 87260	366	121
103	*DNApol*	87378 < 90443	3066	1021	87361 < 90426	3066	1021
104	*desmoplakin*	90448 > 92703	2256	751	90431 > 92686	2256	751
105	*lef-3*	93266 < 94315	1050	349	93249 < 94298	1050	349
106		94281 > 94658	378	125	94264 > 94641	378	125
107		94727 > 95266	540	179	94710 > 95249	540	179
108	*iap-Cp5*	95540 > 96355	816	271	95523 > 96338	816	271
109	*lef-9*	96371 > 97864	1494	497	96354 > 97847	1494	497
110	*fp*	98117 > 98572	456	151	97922 > 98377	456	151
111		98517 > 99008	492	163	98322 > 98813	492	163
112	*DNA ligase*	99106 < 100788	1683	560	98911 < 100593	1683	560
113	*helicase 2*	100892 < 102211	1320	439	100697 < 102016	1320	439
114	*Alk-exo*	102165 < 103364	1200	399	101970 < 103169	1200	399
115		103648 < 103962	315	104	103453 < 103767	315	104
116	*fgf-2*	104102 > 105265	1164	387	103907 > 105070	1164	387
117		105311 < 105511	201	66	105116 < 105316	201	66
118		105587 < 105745	159	52	105392 < 105550	159	52
119	*rr1*	106154 < 107983	1830	609	105959 < 107788	1830	609
120	*rr2a*	108134 > 109171	1038	345	107939 > 108976	1038	345
121	*lef-8*	109360 < 111843	2484	827	109128 < 111611	2484	827
122		112234 > 112635	402	133	112002 > 112403	402	133
123		112773 < 113741	969	**322**	112541 < 113518	978	**325**
124		113813 < 114010	198	65	113590 < 113787	198	65
125	*lef-10*	114014 > 114235	222	73	113791 > 114012	222	73
126	*vp1054*	114099 > 115085	987	328	113876 > 114862	987	328
127		115240 > 115413	174	57	115017 > 115190	174	57
128		115463 > 116488	1026	341	115240 > 116265	1026	341
129	*egt*	116818 < 118122	1305	**434**	116633 < 117985	1353	**450**
130	*me53*	118275 > 119168	894	297	118138 > 119031	894	297

Table S IV 2. List of the SNP positions of PhopGV-R compared to the reference isolate PhopGV-1346. Positions with also the reference sequence supported are highlighted with a star (*).

Number	Positon	Sequence	Nucleotide Change
1	3,135	G	G -> A*
2	3,159	A	A -> T
3	5,475	C	C -> A
4	7,882	T	T -> A*
5	9,514	A	A -> G
6	10,250	C	C -> T
7	10,313	A	A -> G
8	11,262	T	T -> C
9	11,291	T	T -> C
10	11,869	A	A -> G
11	12,811	A	A -> G
12	13,287	T	T -> C
13	13,634	C	C -> T
14	15,219	G	G -> A
15	16,144	T	T -> A
16	16,657	T	T -> G
17	17,725	G	G -> T*
18	17,831	G	G -> A
19	17,957	T	T -> C*
20	18,456	T	T -> A*
21	18,547	G	G -> A
22	19,364	A	A -> G*
23	20,306	C	C -> T*
24	21,377	C	C -> A
25	22,707	T	T -> C
26	23,092	T	T -> C
27	23,766	A	A -> G
28	26,152	G	G -> T
29	26,689	C	C -> G
30	26,880	C	C -> T
31	27,907	C	C -> T
32	28,240	C	C -> T
33	28,469	A	A -> T
34	28,923	T	T -> C
35	29,046	T	T -> C*
36	29,237	G	G -> A
37	32,127	T	T -> C
38	34,630	T	T -> G
39	35,372	G	G -> T*
40	35,905	C	C -> T
41	38,070	T	T -> C
42	41,421	A	A -> T
43	44,853	C	C -> T

44	44,991	C	C -> T
45	45,015	T	T -> C
46	45,394	G	G -> A
47	45,490	C	C -> G*
48	45,581	C	C -> A
49	45,586	A	A -> T
50	45,704	A	A -> G
51	45,827	C	C -> T
52	45,895	C	C -> A
53	46,544	A	A -> G
54	47,389	C	C -> A
55	49,221	G	G -> A
56	50,800	C	C -> A
57	51,733	A	A -> T
58	54,691	T	T -> C
59	56,063	C	C -> T*
60	56,420	A	A -> G*
61	57,537	G	G -> A
62	58,287	C	C -> T
63	65,773	A	A -> C
64	67,084	C	C -> T
65	68,953	C	C -> G
66	69,428	T	T -> C
67	71,916	T	T -> A
68	71,924	A	A -> T
69	75,799	G	G -> A
70	76,009	C	C -> A
71	76,284	C	C -> T
72	76,364	T	T -> C*
73	77,124	T	T -> G
74	78,035	C	C -> T*
75	78,462	G	G -> A*
76	78,683	C	C -> A*
77	79,369	T	T -> C
78	81,581	A	A -> T*
79	85,078	T	T -> G
80	85,232	A	A -> C
81	86,223	T	T -> C
82	86,986	C	C -> T*
83	87,530	G	G -> C
84	89,064	G	G -> A
85	90,684	G	G -> A*
86	93,422	G	G -> C
87	94,066	A	A -> T*
88	94,879	T	T -> C
89	109,726	G	G -> A

90	110,512	C	C -> T
91	110,773	T	T -> A
92	110,863	T	T -> G
93	111,135	C	C -> T
94	112,780	T	T -> C
95	115,269	C	C -> T
96	115,605	A	A -> T
97	116,855	T	T -> C*

Ehrenwörtliche Erklärung:

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit entsprechend den Regeln guter wissenschaftlicher Praxis selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe.

Sämtliche aus fremden Quellen direkt oder indirekt übernommenen Gedanken sowie sämtliche von Anderen direkt oder indirekt übernommenen Daten, Techniken und Materialien sind als solche kenntlich gemacht. Die Arbeit wurde bisher bei keiner anderen Hochschule zu Prüfungszwecken eingereicht.

Darmstadt, den 28. November 2018

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Table S1: Position and orientation of 130 putative ORFs predicted for PhopGV isolates. The ORFs of the different isolates differ to the reference isolate PhopGV-1346 in terms of length or amino acid sequence. The differences are highlighted in bold letters, aa length which differ from the reference and the refering ORFs are underlined. The reading direction is indicated forward (>) and reverse (<).

ORF	Name	PhopGV-1346			PhopGV_SA			CR3.1			CR5.1		
		Position	length (nt)	length (aa)	Position	length (nt)	length (aa)	Position	length (nt)	length (aa)	Position	length (nt)	length (aa)
1	*granulin*	1 > 747	747	248	1 > 747	747	248	1 > 747	747	248	1 > 747	747	248
2	*P78/83*	883 < 1404	522	173	883 < 1404	522	173	883 < 1404	522	173	883 < 1404	522	173
3	*pk-1*	1385 > 2221	837	278	1385 > 2221	837	278	1385 > 2221	837	278	1385 > 2221	837	278
4		2357 < 2929	573	190	2356 < 2928	573	190	2357 < 2929	573	190	2336 < 2908	573	190
5		2922 > 3161	240	79	2921 > 3160	240	79	2922 > 3161	240	79	2901 > 3140	240	79
6	*ie-1*	3538 < 4818	1281	426	3537 < 4817	1,281	426	3538 < 4818	1281	426	3517 < 4797	1281	426
7		4842 > 5459	618	205	4841 > 5458	618	205	4842 > 5459	618	205	4821 > 5438	618	205
8		5479 < 5850	372	123	5478 < 5849	372	123	5479 < 5850	372	123	5458 < 5829	372	123
9	*bro*	6003 > 7079	1077	358	6034 > 7110	1,077	358	6035 > 7111	1077	358	6014 > 7090	1077	358
10		7181 > 7351	171	56	7212 > 7382	171	56	7213 > 7383	171	56	7192 > 7362	171	56
11		7638 > 7877	240	79	7669 > 7908	240	79	7670 > 7909	240	79	7649 > 7888	240	79
12	*ODV-e18*	8027 < 8278	252	83	8058 < 8309	252	83	8059 < 8310	252	83	8038 < 8289	252	83
13	*P49*	8279 < 9559	1281	426	8310 < 9590	1,281	426	8311 < 9591	1281	426	8290 < 9570	1281	426
14		9769 < 10386	618	205	9801 < 10418	618	205	9802 < 10419	618	205	9780 < 10397	618	205
15		10475 < 11434	960	319	10507 < 11466	960	319	10508 < 11467	960	319	10486 < 11445	960	319
16	*ODV-e56*	11464 < 12519	1056	351	11496 < 12551	1,056	351	11497 < 12552	1056	351	11475 < 12530	1056	351
17		12634 > 12822	189	62	12666 > 12854	189	62	12667 > 12855	189	62	12645 > 12833	189	62
18		13124 < 13285	162	**53**	13155 < 13316	162	**53**	13157 < 13318	162	**53**	13135 < 13296	162	**53**
19		13354 < 13827	474	157	13385 < 13858	474	157	13387 < 13860	474	157	13365 < 13838	474	157
20		13890 > 14798	909	302	13921 > 14829	909	302	13923 > 14831	909	302	13901 > 14809	909	302
21		14824 > 15264	441	146	14855 > 15295	441	146	14857 > 15297	441	146	14835 > 15275	441	146
22		15315 > 15680	366	121	15346 > 15711	366	121	15348 > 15713	366	121	15326 > 15691	366	121
23		15754 > 16068	315	104	15785 > 16099	315	104	15787 > 16101	315	104	15765 > 16079	315	104
24	*PE-38*	16369 < 17508	1140	**379**	16460 < 17599	1,140	**379**	16396 < 17535	1140	**379**	16380 < 17519	1140	**379**
25		18497 > 19639	1143	380	18602 > 19744	1,143	380	18548 > 19690	1143	380	18508 > 19650	1143	380
26		20524 > 21063	540	179	20629 > 21168	540	179	20575 > 21114	540	179	20535 > 21074	540	179
27	*efp*	21193 > 22980	1788	595	21296 > 23083	1,788	595	21244 > 23031	1788	595	21204 > 22991	1788	595
28		23479 > 25089	1611	**536**	23558 > 25162	1,605	**534**	23525 > 25120	1596	**531**	23490 > 25100	1611	**536**
29		25295 < 25957	663	220	25331 < 25993	663	220	25290 < 25952	663	220	25306 < 25968	663	220
30		26339 < 26902	564	187	26375 < 26938	564	187	26333 < 26896	564	187	26349 < 26912	564	187
31		26906 > 27439	534	177	26942 > 27475	534	177	26900 > 27433	534	177	26916 > 27449	534	177
32		27436 > 29463	2028	**675**	27472 > 29499	2,028	**675**	27430 > 29457	2028	**675**	27446 > 29473	2028	**675**
33	*ODV-e66*	29470 < 31860	2391	**796**	29506 < 31860	2,355	**784**	29464 < 31830	2367	**788**	29480 < 31846	2367	**788**
34		31911 > 32225	315	104	31911 > 32225	315	104	31881 > 32195	315	104	31897 > 32211	315	104
35		32360 < 32653	294	97	32360 < 32653	294	97	32330 < 32623	294	97	32346 < 32639	294	97
36		32826 > 33161	336	111	32853 > 33188	336	111	32823 > 33158	336	111	32812 > 33147	336	111
37	*lef-2*	33154 > 33696	543	180	33181 > 33723	543	180	33151 > 33693	543	180	33140 > 33682	543	180
38		33951 > 34208	258	85	33978 > 34235	258	85	33948 > 34205	258	85	33937 > 34194	258	85
39		34229 < 34573	345	114	34256 < 34600	345	114	34226 < 34570	345	114	34215 < 34559	345	114
40		34720 < 35073	354	117	34747 < 35100	354	117	34717 < 35070	354	117	34706 < 35059	354	117
41	*mp-nase*	35132 < 36541	1410	469	35159 < 36568	1,410	469	35129 < 36538	1410	469	35118 < 36527	1410	469
42	*p13*	36546 > 37379	834	277	36573 > 37406	834	277	36543 > 37376	834	277	36532 > 37365	834	277
43		37402 > 37716	315	**104**	37429 > 37743	315	**104**	37399 > 37707	309	**102**	37388 > 37702	315	**104**
44		37732 > 38832	1101	366	37759 > 38859	1,101	366	37723 > 38823	1101	366	37718 > 38818	1101	366
45		39075 > 39278	204	67	39103 > 39306	204	67	39067 > 39270	204	67	39062 > 39265	204	67
46		39303 > 41735	2433	**810**	39331 > 41763	2,433	**810**	39295 > 41727	2433	**810**	39290 > 41650	2361	**786**
47		41668 > 42576	909	302	41696 > 42604	909	302	41660 > 42568	909	302	41583 > 42491	909	302
48		42588 > 42731	144	47	42616 > 42759	144	47	42580 > 42723	144	47	42503 > 42646	144	47
49	*v-ubi*	42825 < 43109	285	94	42853 < 43137	285	94	42817 < 43101	285	94	42740 < 43024	285	94
50		43229 > 44248	1020	339	43257 > 44276	1,020	339	43221 > 44240	1020	339	43144 > 44163	1020	339
51		44260 > 44406	147	48	44288 > 44434	147	48	44252 > 44398	147	48	44175 > 44321	147	48
52	*PP31*	44421 < 45116	696	231	44449 < 45144	696	231	44413 < 45108	696	231	44336 < 45031	696	231
53	*lef-11*	45100 < 45366	267	88	45128 < 45394	267	88	45092 < 45358	267	88	45015 < 45281	267	88
54	*sod*	45531 < 45850	390	129	45595 < 45807	213	70	45443 < 45832	390	129	45366 < 45755	390	129
55	*p74*	46284 < 48260	1977	658	46239 < 48215	1,977	658	46266 < 48242	1977	658	46189 < 48165	1977	658
56		48436 < 48852	417	138	48391 < 48807	417	138	48418 < 48834	417	138	48341 < 48757	417	138
57		49042 > 49458	417	138	48997 > 49413	417	138	49024 > 49440	417	138	48947 > 49363	417	138
58		49702 > 50475	774	257	49631 > 50404	774	257	49684 > 50457	774	257	49607 > 50380	774	257
59		50673 < 51266	594	197	50602 < 51195	594	197	50655 < 51248	594	197	50578 < 51171	594	197
60		51354 < 51872	519	172	51283 < 51801	519	172	51336 < 51854	519	172	51259 < 51777	519	172
61	*p47*	51966 > 53165	1200	399	51895 > 53094	1,200	399	51948 > 53147	1200	399	51871 > 53070	1200	399
62		53247 > 53894	648	215	53176 > 53823	648	215	53229 > 53876	648	215	53152 > 53799	648	215
63	*p24*	54103 > 54606	504	167	54032 > 54535	504	167	54085 > 54588	504	167	53980 > 54483	504	167
64		54608 > 55165	558	185	54537 > 55094	558	185	54590 > 55147	558	185	54485 > 55042	558	185
65	*p38.7*	55443 < 56000	558	185	55370 < 55927	558	185	55426 < 55983	558	185	55320 < 55877	558	185
66	*lef-11*	55894 < 56595	702	233	55821 < 56522	702	233	55877 < 56578	702	233	55771 < 56472	702	233
67		56788 > 58356	1569	522	56715 > 58283	1,569	522	56771 > 58339	1569	522	56665 > 58233	1569	522
68		58379 > 58543	165	54	58306 > 58470	165	54	58362 > 58526	165	54	58256 > 58420	165	54
69	*fgf-1*	58591 < 59310	720	239	58518 < 59237	720	239	58574 < 59293	720	239	58468 < 59187	720	239
70		59452 < 59769	318	105	59379 < 59690	312	103	59435 < 59752	318	105	59329 < 59646	318	105
71		60034 > 60492	459	152	59955 > 60413	459	152	60017 > 60475	459	152	59911 > 60369	459	152
72	*lef-6*	60579 < 60863	285	94	60500 < 60784	285	94	60562 < 60846	285	94	60456 < 60740	285	94
73	*dbp*	61039 < 61872	834	277	60941 < 61774	834	277	61022 < 61855	834	277	60874 < 61707	834	277
74		62051 < 62578	528	175	61953 < 62480	528	175	62034 < 62561	528	175	61886 < 62413	528	175
75		62730 > 63869	1140	379	62632 > 63771	1,140	379	62713 > 63852	1140	379	62565 > 63704	1140	379
76		64031 > 64357	327	108	63946 > 64272	327	108	64028 > 64354	327	108	63866 > 64192	327	108
77	*BV/ODV-c42*	64439 > 65578	1140	379	64354 > 65493	1,140	379	64436 > 65575	1140	379	64274 > 65413	1140	379
78	*p6.9*	65595 < 65759	165	54	65510 < 65674	165	54	65592 < 65756	165	54	65430 < 65594	165	54
79	*lef-5*	65908 > 66630	723	240	65823 > 66545	723	240	65906 > 66628	723	240	65743 > 66465	723	240
80		66583 > 67482	900	299	66498 > 67397	900	299	66581 > 67480	900	299	66418 > 67317	900	299
81		67991 < 68479	489	162	67906 < 68394	489	162	67989 < 68477	489	162	67806 < 68294	489	162
82	*helicase 1*	68463 > 71864	3402	1133	68378 > 71779	3,402	1133	68461 > 71862	3402	1133	68278 > 71679	3402	1133
83	*ODV-e25*	72037 < 72681	645	214	71952 < 72596	645	214	72035 < 72679	645	214	71852 < 72496	645	214
84		72812 < 73297	486	161	72727 < 73212	486	161	72810 < 73295	486	161	72627 < 73112	486	161
85		73324 > 74082	759	252	73239 > 73997	759	252	73322 > 74080	759	252	73139 > 73897	759	252
86	*iap-Op1*	74312 < 75049	738	245	74227 < 74964	738	245	74310 < 75047	738	245	74127 < 74864	738	245
87	*lef-4*	75075 < 76400	1326	441	74990 < 76315	1,326	441	75073 < 76398	1326	441	74890 < 76215	1326	441
88	*vp39*	76455 > 77336	882	293	76370 > 77251	882	293	76453 > 77334	882	293	76270 > 77151	882	293
89	*ODV-ec27*	77442 > 78305	864	287	77357 > 78220	864	287	77440 > 78303	864	287	77257 > 78120	864	287
90		78469 > 78621	153	50	78384 > 78536	153	50	78467 > 78619	153	50	78284 > 78436	153	50
91		78629 > 78784	156	51	78544 > 78699	156	51	78627 > 78782	156	51	78444 > 78599	156	51
92		78733 < 79944	1212	403	78648 < 79859	1,212	403	78731 < 79942	1212	403	78548 < 79759	1212	403
93		79931 > 80224	294	97	79846 > 80121	276	91	79929 > 80222	294	97	79746 > 80039	294	97
94	*vp91*	80297 > 82435	2139	**712**	80194 > 82332	2,139	**712**	80295 > 82433	2139	**712**	80112 > 82250	2139	**712**
95	*tlp20*	82410 > 82763	354	117	82289 > 82642	354	117	82408 > 82761	354	117	82225 > 82578	354	117
96		82747 > 83418	672	223	82626 > 83297	672	223	82745 > 83416	672	223	82562 > 83233	672	223
97	*gp41*	83300 > 84157	858	285	83179 > 84036	858	285	83298 > 84155	858	285	83115 > 83972	858	285
98		84423 > 84680	258	85	84302 > 84559	258	85	84421 > 84678	258	85	84238 > 84495	258	85
99	*vlf-1*	84637 > 85782	1146	381	84516 > 85661	1,146	381	84635 > 85780	1146	381	84452 > 85597	1146	381
100		85976 > 86233	258	85	85850 > 86107	258	85	85974 > 86231	258	85	85791 > 86048	258	85
101		86281 > 86718	438	145	86155 > 86592	438	145	86279 > 86716	438	145	86096 > 86533	438	145
102		86912 < 87277	366	121	86772 < 87137	366	121	86910 < 87275	366	121	86727 < 87092	366	121
103	*DNApol*	87378 < 90443	3066	1021	87238 < 90303	3,066	1021	87376 < 90441	3066	1021	87193 < 90258	3066	1021
104	*desmoplakin*	90448 > 92703	2256	**751**	90308 > 92563	2,256	**751**	90446 > 92701	2256	**751**	90263 > 92518	2256	**751**
105	*lef-3*	93266 < 94315	1050	349	93126 < 94175	1,050	349	93264 < 94313	1050	349	93081 < 94130	1050	349
106		94281 > 94658	378	125	94141 > 94518	378	125	94279 > 94656	378	125	94096 > 94473	378	125
107		94727 > 95266	540	179	94587 > 95126	540	179	94725 > 95264	540	179	94542 > 95081	540	179
108	*iap-Cp5*	95540 > 96355	816	271	95400 > 96215	816	271	95538 > 96353	816	271	95355 > 96170	816	271
109	*lef-9*	96371 > 97864	1494	497	96231 > 97724	1,494	497	96369 > 97862	1494	497	96186 > 97679	1494	497
110	*fp*	98117 > 98572	456	151	97799 > 98254	456	151	97937 > 98392	456	151	97754 > 98209	456	151
111		98517 > 99008	492	163	98199 > 98690	492	163	98337 > 98828	492	163	98154 > 98645	492	163
112	*DNA ligase*	99106 < 100788	1683	560	98788 < 100470	1,683	560	98926 < 100608	1683	560	98743 < 100425	1683	560
113	*helicase 2*	100892 < 102211	1320	439	100574 < 101893	1,320	439	100712 < 102031	1320	439	100529 < 101848	1320	439
114	*Alk-exo*	102165 < 103364	1200	399	101847 < 103046	1,200	399	101985 < 103184	1200	399	101802 < 103001	1200	399
115		103648 < 103962	315	104	103376 < 103690	315	104	103468 < 103782	315	104	103285 < 103599	315	104
116	*fgf-2*	104102 > 105265	1164	387	103830 > 104993	1,164	387	103922 > 105085	1164	387	103739 > 104902	1164	387
117		105311 < 105511	201	66	105039 < 105239	201	66	105131 < 105331	201	66	104948 < 105148	201	66
118		105587 < 105745	159	52	105315 < 105473	159	52	105407 < 105565	159	52	105224 < 105382	159	52
119	*rr1*	106154 < 107983	1830	609	105882 < 107711	1,830	609	105974 < 107803	1830	609	105791 < 107620	1830	609
120	*rr2a*	108134 > 109171	1038	345	107862 > 108899	1,038	345	107954 > 108991	1038	345	107771 > 108808	1038	345
121	*lef-8*	109360 < 111843	2484	827	109051 < 111534	2,484	827	109143 < 111626	2484	827	108960 < 111443	2484	827
122		112234 > 112635	402	133	111925 > 112326	402	133	112017 > 112418	402	133	111834 > 112235	402	133
123		112773 < 113741	969	322	112464 < 113441	978	325	112556 < 113533	978	325	112373 < 113350	978	325
124		113813 < 114010	198	65	113513 < 113710	198	65	113605 < 113802	198	65	113422 < 113619	198	65
125	*lef-10*	114014 > 114235	222	73	113714 > 113935	222	73	113806 > 114027	222	73	113623 > 113844	222	73
126	*vp1054*	114099 > 115085	987	328	113799 > 114785	987	328	113891 > 114877	987	328	113708 > 114694	987	328
127		115240 > 115413	174	57	114940 > 115113	174	57	115032 > 115205	174	57	114849 > 115022	174	57
128		115463 > 116488	1026	**341**	115163 > 116188	1,026	**341**	115255 > 116280	1026	**341**	115072 > 116097	1026	**341**
129	*egt*	116818 < 118122	1305	**434**	116556 < 117908	1,353	**450**	116648 < 118000	1353	**450**	116427 < 117731	1305	**434**
130	*me53*	118275 > 119168	894	297	118061 > 118954	894	297	118153 > 119046	894	297	117884 > 118777	894	297

ORF	Name	GR1.1			GR1.2			GR2.1			IT1.1		
		Position	length (nt)	length (aa)	Position	length (nt)	length (aa)	Position	length (nt)	length (aa)	Position	length (nt)	length (aa)
1	*granulin*	1 > 747	747	248	1 > 747	747	248	1 > 747	747	248	1 > 747	747	248
2	*P78/83*	883 < 1404	522	173	883 < 1404	522	173	883 < 1404	522	173	883 < 1404	522	173
3	*pk-1*	1385 > 2221	837	278	1385 > 2221	837	278	1385 > 2221	837	278	1385 > 2221	837	278
4		2357 < 2929	573	190	2357 < 2929	573	190	2357 < 2929	573	190	2357 < 2929	573	190
5		2922 > 3161	240	79	2922 > 3161	240	79	2922 > 3161	240	79	2922 > 3161	240	79
6	*ie-1*	3538 < 4818	1281	426	3538 < 4818	1281	426	3538 < 4818	1281	426	3538 < 4818	1281	426
7		4842 > 5459	618	205	4842 > 5459	618	205	4842 > 5459	618	205	4842 > 5459	618	205
8		5479 < 5850	372	123	5479 < 5850	372	123	5479 < 5850	372	123	5479 < 5850	372	123
9	*bro*	6003 > 7079	1077	358	6003 > 7079	1077	358	6003 > 7079	1077	358	6003 > 7079	1077	358
10		7179 > 7349	171	56	7179 > 7349	171	56	7181 > 7351	171	56	7179 > 7349	171	56
11		7636 > 7875	240	79	7636 > 7875	240	79	7638 > 7877	240	79	7637 > 7876	240	79
12	*ODV-e18*	8025 < 8276	252	83	8025 < 8276	252	83	8027 < 8278	252	83	8026 < 8277	252	83
13	*P49*	8277 < 9557	1281	426	8277 < 9557	1281	426	8279 < 9559	1281	426	8278 < 9558	1281	426
14		9768 < 10385	618	205	9768 < 10385	618	205	9770 < 10387	618	205	9769 < 10386	618	205
15		10474 < 11433	960	319	10474 < 11433	960	319	10476 < 11435	960	319	10475 < 11434	960	319
16	*ODV-e56*	11463 < 12518	1056	351	11463 < 12518	1056	351	11465 < 12520	1056	351	11464 < 12519	1056	351
17		12651 > 12839	189	62	12651 > 12839	189	62	12653 > 12841	189	62	12652 > 12840	189	62
18		13172 < 13327	156	**51**	13172 < 13327	156	**51**	13174 < 13329	156	**51**	13173 < 13328	156	**51**
19		13419 < 13892	474	157	13419 < 13892	474	157	13421 < 13894	474	157	13420 < 13893	474	157
20		13955 > 14863	909	302	13955 > 14863	909	302	13957 > 14865	909	302	13956 > 14864	909	302
21		14889 > 15329	441	146	14889 > 15329	441	146	14891 > 15331	441	146	14890 > 15330	441	146
22		15380 > 15745	366	121	15380 > 15745	366	121	15382 > 15747	366	121	15381 > 15746	366	121
23		15819 > 16133	315	104	15819 > 16133	315	104	15821 > 16135	315	104	15820 > 16134	315	104
24	*PE-38*	16434 < 17573	1140	**379**	16434 < 17573	1140	**379**	16436 < 17575	1140	**379**	16435 < 17574	1140	**379**
25		18590 > 19732	1143	380	18590 > 19732	1143	380	18592 > 19734	1143	380	18591 > 19733	1143	380
26		20617 > 21156	540	179	20617 > 21156	540	179	20619 > 21158	540	179	20618 > 21157	540	179
27	*efp*	21286 > 23073	1788	595	21286 > 23073	1788	595	21288 > 23075	1788	595	21287 > 23074	1788	595
28		23567 > 25177	1611	**536**	23567 > 25177	1611	**536**	23574 > 25184	1611	**536**	23573 > 25183	1611	**536**
29		25364 < 26026	663	220	25364 < 26026	663	220	25407 < 26069	663	220	25406 < 26068	663	220
30		26408 < 26971	564	187	26408 < 26971	564	187	26450 < 27013	564	187	26449 < 27012	564	187
31		26975 > 27508	534	177	26975 > 27508	534	177	27017 > 27550	534	177	27016 > 27549	534	177
32		27505 > 29544	2040	**679**	27505 > 29544	2040	**679**	27547 > 29574	2028	**675**	27546 > 29573	2028	**675**
33	*ODV-e66*	29551 < 31920	2370	**789**	29551 < 31920	2370	**789**	29581 < 31977	2397	**798**	29580 < 31976	2397	**798**
34		31998 > 32312	315	104	31998 > 32312	315	104	32028 > 32342	315	104	32027 > 32341	315	104
35		32447 < 32740	294	97	32447 < 32740	294	97	32477 < 32770	294	97	32476 < 32769	294	97
36		32940 > 33275	336	111	32940 > 33275	336	111	32970 > 33305	336	111	32969 > 33304	336	111
37	*lef-2*	33268 > 33810	543	180	33268 > 33810	543	180	33298 > 33840	543	180	33297 > 33839	543	180
38		34065 > 34322	258	85	34065 > 34322	258	85	34095 > 34352	258	85	34094 > 34351	258	85
39		34343 < 34687	345	114	34343 < 34687	345	114	34373 < 34717	345	114	34372 < 34716	345	114
40		34834 < 35187	354	117	34834 < 35187	354	117	34864 < 35217	354	117	34863 < 35216	354	117
41	*mp-nase*	35246 < 36658	1413	470	35246 < 36658	1413	470	35276 < 36685	1410	469	35275 < 36684	1410	469
42	*p13*	36663 > 37496	834	277	36663 > 37496	834	277	36690 > 37523	834	277	36689 > 37522	834	277
43		37519 > 37833	315	**104**	37519 > 37833	315	**104**	37546 > 37860	315	**104**	37545 > 37859	315	**104**
44		37849 > 38949	1101	366	37849 > 38949	1101	366	37876 > 38976	1101	366	37875 > 38975	1101	366
45		39193 > 39396	204	67	39193 > 39396	204	67	39220 > 39423	204	67	39219 > 39422	204	67
46		39421 > 41823	2403	**800**	39421 > 41823	2403	**800**	39448 > 41868	2421	**806**	39447 > 41849	2403	**800**
47		41756 > 42664	909	302	41756 > 42664	909	302	41801 > 42709	909	302	41782 > 42690	909	302
48		42676 > 42819	144	47	42676 > 42819	144	47	42721 > 42864	144	47	42702 > 42845	144	47
49	*v-ubi*	42913 < 43197	285	94	42913 < 43197	285	94	42958 < 43242	285	94	42939 < 43223	285	94
50		43317 > 44336	1020	339	43317 > 44336	1020	339	43362 > 44381	1020	339	43343 > 44362	1020	339
51		44348 > 44494	147	48	44348 > 44494	147	48	44393 > 44539	147	48	44374 > 44520	147	48
52	*PP31*	44509 < 45204	696	231	44509 < 45204	696	231	44554 < 45249	696	231	44535 < 45230	696	231
53	*lef-11*	45188 < 45454	267	88	45188 < 45454	267	88	45233 < 45499	267	88	45214 < 45480	267	88
54	*sod*	45539 < 45928	390	129	45539 < 45928	390	129	45584 < 45973	390	129	45565 < 45954	390	129
55	*p74*	46362 < 48338	1977	658	46362 < 48338	1977	658	46407 < 48383	1977	658	46388 < 48364	1977	658
56		48514 < 48930	417	138	48514 < 48930	417	138	48559 < 48975	417	138	48540 < 48956	417	138
57		49120 > 49536	417	138	49120 > 49536	417	138	49165 > 49581	417	138	49146 > 49562	417	138
58		49780 > 50553	774	257	49780 > 50553	774	257	49825 > 50598	774	257	49806 > 50579	774	257
59		50751 < 51344	594	197	50751 < 51344	594	197	50796 < 51389	594	197	50777 < 51370	594	197
60		51432 < 51950	519	172	51432 < 51950	519	172	51477 < 51995	519	172	51458 < 51976	519	172
61	*p47*	52044 > 53243	1200	399	52044 > 53243	1200	399	52089 > 53288	1200	399	52070 > 53269	1200	399
62		53325 > 53972	648	215	53325 > 53972	648	215	53370 > 54017	648	215	53351 > 53998	648	215
63	*p24*	54181 > 54684	504	167	54181 > 54684	504	167	54226 > 54729	504	167	54207 > 54710	504	167
64		54686 > 55243	558	185	54686 > 55243	558	185	54731 > 55288	558	185	54712 > 55269	558	185
65	*p38.7*	55521 < 56078	558	185	55521 < 56078	558	185	55566 < 56123	558	185	55547 < 56104	558	185
66	*lef-11*	55972 < 56673	702	233	55972 < 56673	702	233	56017 < 56718	702	233	55998 < 56699	702	233
67		56866 > 58434	1569	522	56866 > 58434	1569	522	56911 > 58479	1569	522	56892 > 58460	1569	522
68		58457 > 58621	165	54	58457 > 58621	165	54	58502 > 58666	165	54	58483 > 58647	165	54
69	*fgf-1*	58669 < 59388	720	239	58669 < 59388	720	239	58714 < 59433	720	239	58695 < 59414	720	239
70		59530 < 59847	318	105	59530 < 59847	318	105	59575 < 59892	318	105	59556 < 59873	318	105
71		60112 > 60570	459	152	60112 > 60570	459	152	60157 > 60615	459	152	60138 > 60596	459	152
72	*lef-6*	60657 < 60941	285	94	60657 < 60941	285	94	60702 < 60986	285	94	60683 < 60967	285	94
73	*dbp*	61075 < 61908	834	277	61075 < 61908	834	277	61160 < 61993	834	277	61141 < 61974	834	277
74		62093 < 62620	528	175	62093 < 62620	528	175	62178 < 62705	528	175	62159 < 62686	528	175
75		62772 > 63911	1140	379	62772 > 63911	1140	379	62857 > 63996	1140	379	62838 > 63977	1140	379
76		64087 > 64413	327	108	64087 > 64413	327	108	64158 > 64484	327	108	64139 > 64465	327	108
77	*BV/ODV-c42*	64495 > 65634	1140	379	64495 > 65634	1140	379	64566 > 65705	1140	379	64547 > 65686	1140	379
78	*p6.9*	65651 < 65815	165	54	65651 < 65815	165	54	65722 < 65886	165	54	65703 < 65867	165	54
79	*lef-5*	65964 > 66686	723	240	65964 > 66686	723	240	66035 > 66757	723	240	66016 > 66738	723	240
80		66639 > 67538	900	299	66639 > 67538	900	299	66710 > 67609	900	299	66691 > 67590	900	299
81		68047 < 68535	489	162	68047 < 68535	489	162	68118 < 68609	492	163	68099 < 68587	489	162
82	*helicase 1*	68519 > 71920	3402	1133	68519 > 71920	3402	1133	68593 > 71994	3402	1133	68571 > 71972	3402	1133
83	*ODV-e25*	72093 < 72737	645	214	72093 < 72737	645	214	72167 < 72811	645	214	72145 < 72789	645	214
84		72868 < 73353	486	161	72868 < 73353	486	161	72942 < 73427	486	161	72920 < 73405	486	161
85		73380 > 74138	759	252	73380 > 74138	759	252	73454 > 74212	759	252	73432 > 74190	759	252
86	*iap-Op1*	74368 < 75105	738	245	74368 < 75105	738	245	74442 < 75179	738	245	74420 < 75157	738	245
87	*lef-4*	75131 < 76456	1326	441	75131 < 76456	1326	441	75205 < 76530	1326	441	75183 < 76508	1326	441
88	*vp39*	76507 > 77388	882	293	76507 > 77388	882	293	76585 > 77466	882	293	76563 > 77444	882	293
89	*ODV-ec27*	77494 > 78357	864	287	77494 > 78357	864	287	77572 > 78435	864	287	77550 > 78413	864	287
90		78521 > 78673	153	50	78521 > 78673	153	50	78599 > 78751	153	50	78577 > 78729	153	50
91		78681 > 78836	156	51	78681 > 78836	156	51	78759 > 78914	156	51	78737 > 78892	156	51
92		78785 < 79996	1212	403	78785 < 79996	1212	403	78863 < 80074	1212	403	78841 < 80052	1212	403
93		79983 > 80276	294	97	79983 > 80276	294	97	80061 > 80354	294	97	80039 > 80332	294	97
94	*vp91*	80349 > 82487	2139	**712**	80349 > 82487	2139	**712**	80427 > 82565	2139	**712**	80405 > 82543	2139	**712**
95	*tlp20*	82462 > 82815	354	117	82462 > 82815	354	117	82540 > 82893	354	117	82518 > 82871	354	117
96		82799 > 83470	672	223	82799 > 83470	672	223	82877 > 83548	672	223	82855 > 83526	672	223
97	*gp41*	83352 > 84209	858	285	83352 > 84209	858	285	83430 > 84287	858	285	83408 > 84265	858	285
98		84475 > 84732	258	85	84475 > 84732	258	85	84553 > 84810	258	85	84531 > 84788	258	85
99	*vlf-1*	84689 > 85834	1146	381	84689 > 85834	1146	381	84767 > 85912	1146	381	84745 > 85890	1146	381
100		86029 > 86286	258	85	86029 > 86286	258	85	86107 > 86364	258	85	86085 > 86342	258	85
101		86334 > 86771	438	145	86334 > 86771	438	145	86412 > 86849	438	145	86390 > 86827	438	145
102		86965 < 87330	366	121	86965 < 87330	366	121	87043 < 87408	366	121	87021 < 87386	366	121
103	*DNApol*	87431 < 90496	3066	1021	87431 < 90496	3066	1021	87509 < 90574	3066	1021	87487 < 90552	3066	1021
104	*desmoplakin*	90501 > 92756	2256	**751**	90501 > 92756	2256	**751**	90579 > 92834	2256	**751**	90557 > 92812	2256	**751**
105	*lef-3*	93319 < 94368	1050	349	93319 < 94368	1050	349	93397 < 94446	1050	349	93375 < 94424	1050	349
106		94334 > 94711	378	125	94334 > 94711	378	125	94412 > 94789	378	125	94390 > 94767	378	125
107		94780 > 95319	540	179	94780 > 95319	540	179	94858 > 95397	540	179	94836 > 95375	540	179
108	*iap-Cp5*	95593 > 96408	816	271	95593 > 96408	816	271	95671 > 96486	816	271	95649 > 96464	816	271
109	*lef-9*	96424 > 97917	1494	497	96424 > 97917	1494	497	96502 > 97995	1494	497	96480 > 97973	1494	497
110	*fp*	98166 > 98621	456	151	98166 > 98621	456	151	98244 > 98699	456	151	98222 > 98677	456	151
111		98566 > 99057	492	163	98566 > 99057	492	163	98644 > 99135	492	163	98622 > 99113	492	163
112	*DNA ligase*	99155 < 100837	1683	560	99155 < 100837	1683	560	99233 < 100915	1683	560	99211 < 100893	1683	560
113	*helicase 2*	100941 < 102260	1320	439	100941 < 102260	1320	439	101019 < 102338	1320	439	100997 < 102316	1320	439
114	*Alk-exo*	102214 < 103413	1200	399	102214 < 103413	1200	399	102292 < 103491	1200	399	102270 < 103469	1200	399
115		103697 < 104011	315	104	103697 < 104011	315	104	103775 < 104089	315	104	103753 < 104067	315	104
116	*fgf-2*	104151 > 105314	1164	387	104151 > 105314	1164	387	104229 > 105392	1164	387	104207 > 105370	1164	387
117		105360 < 105560	201	66	105360 < 105560	201	66	105438 < 105638	201	66	105416 < 105616	201	66
118		105636 < 105794	159	52	105636 < 105794	159	52	105714 < 105872	159	52	105692 < 105850	159	52
119	*rr1*	106203 < 108032	1830	609	106203 < 108032	1830	609	106281 < 108110	1830	609	106259 < 108088	1830	609
120	*rr2a*	108183 > 109220	1038	345	108183 > 109220	1038	345	108261 > 109298	1038	345	108239 > 109276	1038	345
121	*lef-8*	109409 < 111892	2484	827	109409 < 111892	2484	827	109487 < 111970	2484	827	109465 < 111948	2484	827
122		112283 > 112684	402	133	112283 > 112684	402	133	112361 > 112762	402	133	112339 > 112740	402	133
123		112822 < 113799	978	325	112822 < 113799	978	325	112900 < 113877	978	325	112878 < 113855	978	325
124		113871 < 114068	198	65	113871 < 114068	198	65	113949 < 114146	198	65	113927 < 114124	198	65
125	*lef-10*	114072 > 114293	222	73	114072 > 114293	222	73	114150 > 114371	222	73	114128 > 114349	222	73
126	*vp1054*	114157 > 115143	987	328	114157 > 115143	987	328	114235 > 115221	987	328	114213 > 115199	987	328
127		115298 > 115471	174	57	115298 > 115471	174	57	115376 > 115549	174	57	115354 > 115527	174	57
128		115521 > 116546	1026	**341**	115521 > 116546	1026	**341**	115599 > 116624	1026	**341**	115577 > 116602	1026	**341**
129	*egt*	116605 < 117966	1362	**453**	116605 < 117966	1362	**453**	116746 < 118044	1299	**432**	116724 < 118022	1299	**432**
130	*me53*	118119 > 119012	894	297	118119 > 119012	894	297	118197 > 119090	894	297	118175 > 119068	894	297

ORF	Name	LS1.1			LS1.2			LS2.1			LS3.1		
		Position	length (nt)	length (aa)	Position	length (nt)	length (aa)	Position	length (nt)	length (aa)	Position	length (nt)	length (aa)
1	*granulin*	1 > 747	747	248	1 > 747	747	248	1 > 747	747	248	1 > 747	747	248
2	*P78/83*	883 < 1404	522	173	883 < 1404	522	173	883 < 1404	522	173	883 < 1404	522	173
3	*pk-1*	1385 > 2221	837	278	1385 > 2221	837	278	1385 > 2221	837	278	1385 > 2221	837	278
4		2386 < 2958	573	190	2356 < 2928	573	190	2357 < 2929	573	190	2386 < 2958	573	190
5		2951 > 3190	240	79	2921 > 3160	240	79	2922 > 3161	240	79	2951 > 3190	240	79
6	*ie-1*	3567 < 4847	1281	426	3537 < 4817	1281	426	3538 < 4818	1281	426	3567 < 4847	1281	426
7		4871 > 5488	618	205	4841 > 5458	618	205	4842 > 5459	618	205	4871 > 5488	618	205
8		5508 < 5879	372	123	5478 < 5849	372	123	5479 < 5850	372	123	5508 < 5879	372	123
9	*bro*	6064 > 7140	1077	358	6034 > 7110	1077	358	6035 > 7111	1077	358	6064 > 7140	1077	358
10		7242 > 7412	171	56	7212 > 7382	171	56	7213 > 7383	171	56	7242 > 7412	171	56
11		7699 > 7938	240	79	7669 > 7908	240	79	7670 > 7909	240	79	7699 > 7938	240	79
12	*ODV-e18*	8088 < 8339	252	83	8058 < 8309	252	83	8062 < 8313	252	83	8088 < 8339	252	83
13	*P49*	8340 < 9620	1281	426	8310 < 9590	1281	426	8314 < 9594	1281	426	8340 < 9620	1281	426
14		9831 < 10448	618	205	9801 < 10418	618	205	9805 < 10422	618	205	9831 < 10448	618	205
15		10537 < 11496	960	319	10507 < 11466	960	319	10511 < 11470	960	319	10537 < 11496	960	319
16	*ODV-e56*	11526 < 12581	1056	351	11496 < 12551	1056	351	11500 < 12555	1056	351	11526 < 12581	1056	351
17		12696 > 12884	189	62	12666 > 12854	189	62	12670 > 12858	189	62	12696 > 12884	189	62
18		13186 < 13347	162	**53**	13156 < 13317	162	**53**	13160 < 13321	162	**53**	13186 < 13347	162	**53**
19		13416 < 13889	474	157	13386 < 13859	474	157	13390 < 13863	474	157	13416 < 13889	474	157
20		13952 > 14860	909	302	13922 > 14830	909	302	13926 > 14834	909	302	13952 > 14860	909	302
21		14886 > 15326	441	146	14856 > 15296	441	146	14860 > 15300	441	146	14886 > 15326	441	146
22		15377 > 15742	366	121	15347 > 15712	366	121	15351 > 15716	366	121	15377 > 15742	366	121
23		15816 > 16130	315	104	15786 > 16100	315	104	15790 > 16104	315	104	15816 > 16130	315	104
24	*PE-38*	16412 < 17548	1137	**378**	16381 < 17517	1137	**378**	16363 < 17502	1140	**379**	16434 < 17570	1137	**378**
25		18580 > 19722	1143	380	18530 > 19672	1143	380	18461 > 19603	1143	380	18583 > 19725	1143	380
26		20607 > 21146	540	179	20557 > 21096	540	179	20488 > 21027	540	179	20610 > 21149	540	179
27	*efp*	21276 > 23063	1788	595	21226 > 23013	1788	595	21157 > 22944	1788	595	21279 > 23066	1788	595
28		23557 > 25152	1596	**531**	23507 > 25102	1596	**531**	23403 > 25013	1611	**536**	23550 > 25145	1596	**531**
29		25358 < 26020	663	220	25272 < 25934	663	220	25183 < 25845	663	220	25315 < 25977	663	220
30		26401 < 26964	564	187	26315 < 26878	564	187	26227 < 26790	564	187	26358 < 26921	564	187
31		26968 > 27501	534	177	26882 > 27415	534	177	26794 > 27327	534	177	26925 > 27458	534	177
32		27498 > 29525	2028	**675**	27412 > 29439	2028	**675**	27324 > 29348	2025	**674**	27455 > 29482	2028	**675**
33	*ODV-e66*	29532 < 31898	2367	**788**	29446 < 31812	2367	**788**	29355 < 31712	2358	**785**	29489 < 31843	2355	**784**
34		31949 > 32263	315	104	31863 > 32177	315	104	31790 > 32104	315	104	31894 > 32208	315	104
35		32398 < 32691	294	97	32312 < 32605	294	97	32239 < 32532	294	97	32343 < 32636	294	97
36		32891 > 33226	336	111	32805 > 33140	336	111	32732 > 33067	336	111	32836 > 33171	336	111
37	*lef-2*	33219 > 33761	543	180	33133 > 33675	543	180	33060 > 33602	543	180	33164 > 33706	543	180
38		34016 > 34273	258	85	33930 > 34187	258	85	33857 > 34114	258	85	33961 > 34218	258	85
39		34294 < 34638	345	114	34208 < 34552	345	114	34135 < 34479	345	114	34239 < 34583	345	114
40		34785 < 35138	354	117	34699 < 35052	354	117	34626 < 34979	354	117	34730 < 35083	354	117
41	*mp-nase*	35197 < 36606	1410	469	35111 < 36520	1410	469	35038 < 36447	1410	469	35142 < 36551	1410	469
42	*p13*	36611 > 37444	834	277	36525 > 37358	834	277	36452 > 37285	834	277	36556 > 37389	834	277
43		37467 > 37775	309	**102**	37381 > 37689	309	**102**	37308 > 37622	315	**104**	37412 > 37720	309	**102**
44		37791 > 38891	1101	366	37705 > 38805	1101	366	37638 > 38738	1101	366	37736 > 38836	1101	366
45		39135 > 39338	204	67	39049 > 39252	204	67	38954 > 39157	204	67	39080 > 39283	204	67
46		39363 > 41795	2433	**810**	39277 > 41679	2403	**800**	39182 > 41602	2421	**806**	39308 > 41740	2433	**810**
47		41728 > 42636	909	302	41612 > 42520	909	302	41520 > 42428	909	302	41673 > 42581	909	302
48		42648 > 42791	144	47	42532 > 42675	144	47	42440 > 42583	144	47	42593 > 42736	144	47
49	*v-ubi*	42885 < 43169	285	94	42769 < 43053	285	94	42677 < 42961	285	94	42830 < 43114	285	94
50		43289 > 44308	1020	339	43173 > 44192	1020	339	43081 > 44100	1020	339	43234 > 44253	1020	339
51		44320 > 44466	147	48	44204 > 44350	147	48	44112 > 44258	147	48	44265 > 44411	147	48
52	*PP31*	44481 < 45176	696	231	44365 < 45060	696	231	44273 < 44968	696	231	44426 < 45121	696	231
53	*lef-11*	45160 < 45426	267	88	45044 < 45310	267	88	44952 < 45218	267	88	45105 < 45371	267	88
54	*sod*	45822 < 45899	78	25	45706 < 45783	78	25	45320 < 45691	372	123	45767 < 45844	78	25
55	*p74*	46333 < 48309	1977	658	46217 < 48193	1977	658	46125 < 48101	1977	658	46278 < 48254	1977	658
56		48485 < 48901	417	138	48369 < 48785	417	138	48276 < 48692	417	138	48430 < 48846	417	138
57		49091 > 49507	417	138	48975 > 49391	417	138	48882 > 49298	417	138	49036 > 49452	417	138
58		49751 > 50524	774	257	49635 > 50408	774	257	49490 > 50263	774	257	49696 > 50469	774	257
59		50722 < 51315	594	197	50606 < 51199	594	197	50461 < 51054	594	197	50667 < 51260	594	197
60		51403 < 51921	519	172	51287 < 51805	519	172	51142 < 51660	519	172	51348 < 51866	519	172
61	*p47*	52015 > 53214	1200	399	51899 > 53098	1200	399	51754 > 52953	1200	399	51960 > 53159	1200	399
62		53296 > 53943	648	215	53180 > 53827	648	215	53035 > 53682	648	215	53241 > 53888	648	215
63	*p24*	54152 > 54655	504	167	54036 > 54539	504	167	53891 > 54394	504	167	54097 > 54600	504	167
64		54657 > 55214	558	185	54541 > 55098	558	185	54396 > 54953	558	185	54602 > 55159	558	185
65	*p38.7*	55493 < 56050	558	185	55377 < 55934	558	185	55232 < 55789	558	185	55438 < 55995	558	185
66	*lef-11*	55944 < 56645	702	233	55828 < 56529	702	233	55683 < 56384	702	233	55889 < 56590	702	233
67		56838 > 58406	1569	522	56722 > 58290	1569	522	56578 > 58146	1569	522	56783 > 58351	1569	522
68		58429 > 58593	165	54	58313 > 58477	165	54	58169 > 58333	165	54	58374 > 58538	165	54
69	*fgf-1*	58641 < 59360	720	239	58525 < 59244	720	239	58381 < 59100	720	239	58586 < 59305	720	239
70		59502 < 59819	318	105	59386 < 59703	318	105	59242 < 59559	318	105	59447 < 59764	318	105
71		60084 > 60542	459	152	59968 > 60426	459	152	59824 > 60282	459	152	60029 > 60487	459	152
72	*lef-6*	60629 < 60913	285	94	60513 < 60797	285	94	60369 < 60653	285	94	60574 < 60858	285	94
73	*dbp*	61089 < 61922	834	277	60973 < 61806	834	277	60829 < 61662	834	277	61034 < 61867	834	277
74		62101 < 62628	528	175	61985 < 62512	528	175	61841 < 62368	528	175	62046 < 62573	528	175
75		62780 > 63919	1140	379	62685 > 63824	1140	379	62520 > 63659	1140	379	62725 > 63864	1140	379
76		64091 > 64417	327	108	63996 > 64322	327	108	63834 > 64160	327	108	64036 > 64362	327	108
77	*BV/ODV-c42*	64499 > 65638	1140	379	64404 > 65543	1140	379	64242 > 65381	1140	379	64444 > 65583	1140	379
78	*p6.9*	65655 < 65819	165	54	65560 < 65724	165	54	65398 < 65562	165	54	65600 < 65764	165	54
79	*lef-5*	65968 > 66690	723	240	65873 > 66595	723	240	65711 > 66433	723	240	65913 > 66635	723	240
80		66643 > 67542	900	299	66548 > 67447	900	299	66386 > 67285	900	299	66588 > 67487	900	299
81		68051 < 68539	489	162	67956 < 68444	489	162	67794 < 68282	489	162	67996 < 68484	489	162
82	*helicase 1*	68523 > 71924	3402	1133	68428 > 71829	3402	1133	68266 > 71667	3402	1133	68468 > 71869	3402	1133
83	*ODV-e25*	72097 < 72741	645	214	72002 < 72646	645	214	71824 < 72468	645	214	72042 < 72686	645	214
84		72872 < 73357	486	161	72777 < 73262	486	161	72599 < 73084	486	161	72817 < 73302	486	161
85		73384 > 74142	759	252	73289 > 74047	759	252	73111 > 73869	759	252	73329 > 74087	759	252
86	*iap-Op1*	74372 < 75109	738	245	74277 < 75014	738	245	74099 < 74836	738	245	74317 < 75054	738	245
87	*lef-4*	75135 < 76460	1326	441	75040 < 76365	1326	441	74862 < 76187	1326	441	75080 < 76405	1326	441
88	*vp39*	76515 > 77396	882	293	76420 > 77301	882	293	76242 > 77123	882	293	76460 > 77341	882	293
89	*ODV-ec27*	77502 > 78365	864	287	77407 > 78270	864	287	77229 > 78092	864	287	77447 > 78310	864	287
90		78529 > 78681	153	50	78434 > 78586	153	50	78256 > 78408	153	50	78474 > 78626	153	50
91		78689 > 78844	156	51	78594 > 78749	156	51	78416 > 78571	156	51	78634 > 78789	156	51
92		78793 < 80004	1212	403	78698 < 79909	1212	403	78520 < 79731	1212	403	78738 < 79949	1212	403
93		79991 > 80284	294	97	79896 > 80189	294	97	79718 > 80011	294	97	79936 > 80229	294	97
94	*vp91*	80357 > 82495	2139	**712**	80262 > 82400	2139	**712**	80084 > 82241	2157	**718**	80302 > 82440	2139	**712**
95	*tlp20*	82470 > 82823	354	117	82375 > 82728	354	117	82215 > 82568	354	117	82415 > 82768	354	117
96		82807 > 83478	672	223	82712 > 83383	672	223	82552 > 83223	672	223	82752 > 83423	672	223
97	*gp41*	83360 > 84217	858	285	83265 > 84122	858	285	83105 > 83962	858	285	83305 > 84162	858	285
98		84483 > 84740	258	85	84388 > 84645	258	85	84228 > 84485	258	85	84428 > 84685	258	85
99	*vlf-1*	84697 > 85842	1146	381	84602 > 85747	1146	381	84442 > 85587	1146	381	84642 > 85787	1146	381
100		86036 > 86293	258	85	85941 > 86198	258	85	85781 > 86038	258	85	85981 > 86238	258	85
101		86341 > 86778	438	145	86246 > 86683	438	145	86086 > 86523	438	145	86286 > 86723	438	145
102		86972 < 87337	366	121	86862 < 87227	366	121	86717 < 87082	366	121	86917 < 87282	366	121
103	*DNApol*	87438 < 90503	3066	1021	87328 < 90393	3066	1021	87183 < 90248	3066	1021	87383 < 90448	3066	1021
104	*desmoplakin*	90508 > 92763	2256	**751**	90398 > 92653	2256	**751**	90253 > 92463	2211	**736**	90453 > 92708	2256	**751**
105	*lef-3*	93326 < 94375	1050	349	93216 < 94265	1050	349	93026 < 94075	1050	349	93271 < 94320	1050	349
106		94341 > 94718	378	125	94231 > 94608	378	125	94041 > 94418	378	125	94286 > 94663	378	125
107		94787 > 95326	540	179	94677 > 95216	540	179	94487 > 95026	540	179	94732 > 95271	540	179
108	*iap-Cp5*	95600 > 96415	816	271	95490 > 96305	816	271	95275 > 96090	816	271	95545 > 96360	816	271
109	*lef-9*	96431 > 97924	1494	497	96321 > 97814	1494	497	96106 > 97599	1494	497	96376 > 97869	1494	497
110	*fp*	97999 > 98454	456	151	97889 > 98344	456	151	97674 > 98129	456	151	97944 > 98399	456	151
111		98399 > 98890	492	163	98289 > 98780	492	163	98074 > 98565	492	163	98344 > 98835	492	163
112	*DNA ligase*	98988 < 100670	1683	560	98878 < 100560	1683	560	98663 < 100345	1683	560	98933 < 100615	1683	560
113	*helicase 2*	100774 < 102093	1320	439	100664 < 101983	1320	439	100449 < 101768	1320	439	100719 < 102038	1320	439
114	*Alk-exo*	102047 < 103246	1200	399	101937 < 103136	1200	399	101722 < 102921	1200	399	101992 < 103191	1200	399
115		103530 < 103844	315	104	103420 < 103734	315	104	103251 < 103565	315	104	103475 < 103789	315	104
116	*fgf-2*	103984 > 105147	1164	387	103874 > 105037	1164	387	103705 > 104868	1164	387	103929 > 105092	1164	387
117		105193 < 105393	201	66	105083 < 105283	201	66	104914 < 105114	201	66	105138 < 105338	201	66
118		105469 < 105627	159	52	105359 < 105517	159	52	105190 < 105348	159	52	105414 < 105572	159	52
119	*rr1*	106036 < 107865	1830	609	105926 < 107755	1830	609	105757 < 107586	1830	609	105981 < 107810	1830	609
120	*rr2a*	108016 > 109053	1038	345	107906 > 108943	1038	345	107737 > 108774	1038	345	107961 > 108998	1038	345
121	*lef-8*	109205 < 111688	2484	827	109095 < 111578	2484	827	108929 < 111412	2484	827	109150 < 111633	2484	827
122		112079 > 112480	402	133	111969 > 112370	402	133	111803 > 112204	402	133	112024 > 112425	402	133
123		112618 < 113595	978	325	112508 < 113485	978	325	112342 < 113319	978	325	112563 < 113540	978	325
124		113667 < 113864	198	65	113557 < 113754	198	65	113391 < 113588	198	65	113612 < 113809	198	65
125	*lef-10*	113868 > 114089	222	73	113758 > 113979	222	73	113592 > 113813	222	73	113813 > 114034	222	73
126	*vp1054*	113953 > 114939	987	328	113843 > 114829	987	328	113677 > 114663	987	328	113898 > 114884	987	328
127		115094 > 115267	174	57	114984 > 115157	174	57	114818 > 114991	174	57	115039 > 115212	174	57
128		115317 > 116342	1026	**341**	115207 > 116232	1026	**341**	115023 > 116031	1008	**335**	115262 > 116287	1026	**341**
129	*egt*	116710 < 118062	1353	**450**	116600 < 117952	1353	**450**	116416 < 117768	1353	**450**	116655 < 118007	1353	**450**
130	*me53*	118215 > 119108	894	297	118105 > 118998	894	297	117921 > 118814	894	297	118160 > 119053	894	297

ORF	Name	YM.1			R		
		Position	length (nt)	length (aa)	Position	length (nt)	length (aa)
1	*granulin*	1 > 747	747	248	1 > 747	747	248
2	*P78/83*	883 < 1404	522	173	883 < 1404	522	173
3	*pk-1*	1385 > 2221	837	278	1385 > 2221	837	278
4		2357 < 2929	573	190	2386 < 2958	573	190
5		2922 > 3161	240	79	2951 > 3190	240	79
6	*ie-1*	3538 < 4818	1281	426	3567 < 4847	1281	426
7		4842 > 5459	618	205	4871 > 5488	618	205
8		5479 < 5850	372	123	5508 < 5879	372	123
9	*bro*	6035 > 7111	1077	358	6064 > 7140	1077	358
10		7213 > 7383	171	56	7242 > 7412	171	56
11		7670 > 7909	240	79	7699 > 7938	240	79
12	*ODV-e18*	8059 < 8310	252	83	8088 < 8339	252	83
13	*P49*	8311 < 9591	1281	426	8340 < 9620	1281	426
14		9802 < 10419	618	205	9831 < 10448	618	205
15		10508 < 11467	960	319	10529 < 11488	960	319
16	*ODV-e56*	11497 < 12552	1056	351	11518 < 12573	1056	351
17		12667 > 12855	189	62	12688 > 12876	189	62
18		13157 < 13318	162	**53**	13178 < 13339	162	**53**
19		13387 < 13860	474	157	13408 < 13881	474	157
20		13923 > 14831	909	302	13944 > 14852	909	302
21		14857 > 15297	441	146	14878 > 15318	441	146
22		15348 > 15713	366	121	15369 > 15734	366	121
23		15787 > 16101	315	104	15808 > 16122	315	104
24	*PE-38*	16400 < 17539	1140	**379**	16423 < 17559	1137	**378**
25		18479 > 19621	1143	380	18546 > 19688	1143	380
26		20506 > 21045	540	179	20573 > 21112	540	179
27	*efp*	21175 > 22962	1788	595	21242 > 23029	1788	595
28		23428 > 25038	1611	**536**	23528 > 25123	1596	**531**
29		25208 < 25870	663	220	25293 < 25955	663	220
30		26251 < 26814	564	187	26336 < 26899	564	187
31		26818 > 27351	534	177	26903 > 27436	534	177
32		27348 > 29372	2025	**674**	27433 > 29460	2028	**675**
33	*ODV-e66*	29379 < 31739	2361	**786**	29467 < 31833	2367	**788**
34		31790 > 32104	315	104	31884 > 32198	315	104
35		32239 < 32532	294	97	32333 < 32626	294	97
36		32732 > 33067	336	111	32826 > 33161	336	111
37	*lef-2*	33060 > 33602	543	180	33154 > 33696	543	180
38		33857 > 34114	258	85	33951 > 34208	258	85
39		34135 < 34479	345	114	34229 < 34573	345	114
40		34626 < 34979	354	117	34720 < 35073	354	117
41	*mp-nase*	35038 < 36447	1410	469	35132 < 36541	1410	469
42	*p13*	36452 > 37285	834	277	36546 > 37379	834	277
43		37308 > 37622	315	**104**	37402 > 37710	309	**102**
44		37638 > 38738	1101	366	37726 > 38826	1101	366
45		38954 > 39157	204	67	39070 > 39273	204	67
46		39182 > 41590	2409	**802**	39298 > 41730	2433	**810**
47		41523 > 42431	909	302	41663 > 42571	909	302
48		42443 > 42586	144	47	42583 > 42726	144	47
49	*v-ubi*	42680 < 42964	285	94	42820 < 43104	285	94
50		43084 > 44103	1020	339	43224 > 44243	1020	339
51		44115 > 44261	147	48	44255 > 44401	147	48
52	*PP31*	44276 < 44971	696	231	44416 < 45111	696	231
53	*lef-11*	44955 < 45221	267	88	45095 < 45361	267	88
54	*sod*	45323 < 45694	372	123	45446 < 45835	390	129
55	*p74*	46128 < 48104	1977	658	46270 < 48246	1977	658
56		48280 < 48696	417	138	48422 < 48838	417	138
57		48886 > 49302	417	138	49028 > 49444	417	138
58		49546 > 50319	774	257	49688 > 50461	774	257
59		50517 < 51110	594	197	50659 < 51252	594	197
60		51198 < 51716	519	172	51340 < 51858	519	172
61	*p47*	51810 > 53009	1200	399	51952 > 53151	1200	399
62		53091 > 53738	648	215	53233 > 53880	648	215
63	*p24*	53947 > 54450	504	167	54089 > 54592	504	167
64		54452 > 55009	558	185	54594 > 55151	558	185
65	*p38.7*	55288 < 55845	558	185	55430 < 55987	558	185
66	*lef-11*	55739 < 56440	702	233	55881 < 56582	702	233
67		56633 > 58201	1569	522	56775 > 58343	1569	522
68		58224 > 58463	240	79	58366 > 58530	165	54
69	*fgf-1*	58437 < 59156	720	239	58578 < 59297	720	239
70		59298 < 59609	312	103	59439 < 59756	318	105
71		59874 > 60332	459	152	60021 > 60479	459	152
72	*lef-6*	60419 < 60703	285	94	60566 < 60850	285	94
73	*dbp*	60879 < 61712	834	277	61026 < 61859	834	277
74		61891 < 62418	528	175	62038 < 62565	528	175
75		62570 > 63709	1140	379	62717 > 63856	1140	379
76		63885 > 64211	327	108	64032 > 64358	327	108
77	*BV/ODV-c42*	64293 > 65432	1140	379	64440 > 65579	1140	379
78	*p6.9*	65449 < 65613	165	54	65596 < 65760	165	54
79	*lef-5*	65762 > 66484	723	240	65910 > 66632	723	240
80		66437 > 67336	900	299	66585 > 67484	900	299
81		67845 < 68333	489	162	67993 < 68481	489	162
82	*helicase 1*	68317 > 71718	3402	1133	68465 > 71866	3402	1133
83	*ODV-e25*	71875 < 72519	645	214	72039 < 72683	645	214
84		72650 < 73135	486	161	72814 < 73299	486	161
85		73162 > 73920	759	252	73326 > 74084	759	252
86	*iap-Op1*	74150 < 74887	738	245	74313 < 75050	738	245
87	*lef-4*	74913 < 76238	1326	441	75076 < 76401	1326	441
88	*vp39*	76293 > 77174	882	293	76456 > 77337	882	293
89	*ODV-ec27*	77280 > 78143	864	287	77443 > 78306	864	287
90		78307 > 78459	153	50	78470 > 78622	153	50
91		78467 > 78622	156	51	78630 > 78785	156	51
92		78571 < 79782	1212	403	78734 < 79945	1212	403
93		79769 > 80062	294	97	79932 > 80225	294	97
94	*vp91*	80135 > 82291	2157	**718**	80298 > 82418	2121	**706**
95	*tlp20*	82266 > 82619	354	117	82393 > 82746	354	117
96		82603 > 83274	672	223	82730 > 83401	672	223
97	*gp41*	83156 > 84013	858	285	83283 > 84140	858	285
98		84279 > 84536	258	85	84406 > 84663	258	85
99	*vlf-1*	84493 > 85638	1146	381	84620 > 85765	1146	381
100		85832 > 86089	258	85	85959 > 86216	258	85
101		86137 > 86574	438	145	86264 > 86701	438	145
102		86768 < 87133	366	121	86895 < 87260	366	121
103	*DNApol*	87234 < 90299	3066	1021	87361 < 90426	3066	1021
104	*desmoplakin*	90304 > 92514	2211	**736**	90431 > 92686	2256	**751**
105	*lef-3*	93077 < 94126	1050	349	93249 < 94298	1050	349
106		94092 > 94469	378	125	94264 > 94641	378	125
107		94538 > 95077	540	179	94710 > 95249	540	179
108	*iap-Cp5*	95351 > 96166	816	271	95523 > 96338	816	271
109	*lef-9*	96182 > 97675	1494	497	96354 > 97847	1494	497
110	*fp*	97750 > 98205	456	151	97922 > 98377	456	151
111		98150 > 98641	492	163	98322 > 98813	492	163
112	*DNA ligase*	98739 < 100421	1683	560	98911 < 100593	1683	560
113	*helicase 2*	100525 < 101844	1320	439	100697 < 102016	1320	439
114	*Alk-exo*	101798 < 102997	1200	399	101970 < 103169	1200	399
115		103327 < 103641	315	104	103453 < 103767	315	104
116	*fgf-2*	103781 > 104944	1164	387	103907 > 105070	1164	387
117		104990 < 105190	201	66	105116 < 105316	201	66
118		105266 < 105424	159	52	105392 < 105550	159	52
119	*rr1*	105833 < 107662	1830	609	105959 < 107788	1830	609
120	*rr2a*	107813 > 108850	1038	345	107939 > 108976	1038	345
121	*lef-8*	109002 < 111485	2484	827	109128 < 111611	2484	827
122		111876 > 112277	402	133	112002 > 112403	402	133
123		112415 < 113392	978	325	112541 < 113518	978	325
124		113464 < 113661	198	65	113590 < 113787	198	65
125	*lef-10*	113665 > 113886	222	73	113791 > 114012	222	73
126	*vp1054*	113750 > 114736	987	328	113876 > 114862	987	328
127		114891 > 115064	174	57	115017 > 115190	174	57
128		115114 > 116121	1008	**335**	115240 > 116265	1026	**341**
129	*egt*	116489 < 117841	1353	**450**	116633 < 117985	1353	**450**
130	*me53*	117994 > 118887	894	297	118138 > 119031	894	297

Table S2: All SNP positions and appropriate frequencies of the sequenced PhopGV isolates in relation to the reference isolate PhopGV-1346. Phred-scaled probability of all samples being homozygous to reference (QUAL); read depth (DP); number of reads covering reference forward, ref. reverse, alternative for. and alt. rev. (DP4); read depth of different alleles for reference, alternative 1, alt. 2 and alt. 3 (DPR) and genotype quality value (GQ) are indicated.

POSITION	REF	ALT	QUAL	CR3.1				CR5.1				GR1.1			
				DP	DP4	DPR	GQ	DP	DP4	DPR	GQ	DP	DP4	DPR	GQ
723	T	G.A.C	215	481	336,141,3,1	477,2,0,2	127	511	377,134,0,0	511,0,0,0	127	517	344,172,1,0	516,0,1,0	127
952	C	T.A.G	198	541	219,320,1,1	539,1,1,0	127	547	200,345,1,1	545,1,0,1	127	535	229,303,2,1	532,0,3,0	127
974	G	T.A.C	674	531	224,307,0,0	531,0,0,0	127	535	205,329,1,0	534,1,0,0	127	536	239,289,4,4	528,6,2,0	127
1033	C	T.A.G	46	510	188,317,2,3	505,1,4,0	127	507	180,322,3,2	502,1,3,1	127	500	213,286,1,0	499,0,1,0	127
1351	G	T.A.C	999	515	224,212,37,42	436,69,10,0	42	515	199,231,43,42	430,50,35,0	127	498	3,4,246,245	7,489,2,0	127
1418	C	A.T.G	198	499	323,175,0,1	498,1,0,0	127	519	316,200,2,1	516,2,1,0	127	508	288,218,1,1	506,2,0,0	127
1762	T	C.A.G	999	511	231,246,19,15	477,31,2,1	127	539	243,257,16,23	500,37,2,0	127	494	3,3,232,256	6,488,0,0	127
2289	T	A.C.G	19	200	115,84,0,1	199,1,0,0	127	122	56,66,0,0	122,0,0,0	127	464	259,205,0,0	464,0,0,0	127
2627	T	C.A.G	999	483	262,175,27,19	437,45,1,0	127	520	237,240,26,17	477,41,1,1	127	474	6,7,262,199	13,460,1,0	127
2660	C	T.A.G	999	498	242,213,21,22	455,43,0,0	127	522	247,254,6,15	501,17,2,2	127	431	8,7,224,192	15,414,1,1	127
2821	G	T.C.A	26	497	327,126,32,12	453,43,1,0	127	510	356,152,1,1	508,1,0,1	127	456	328,128,0,0	456,0,0,0	127
3135	G	A.T.C	912	530	152,203,75,100	355,172,3,0	127	556	215,236,50,55	451,104,1,0	73	492	194,293,3,2	487,2,3,0	127
3159	A	T.G.C	999	499	1,0,177,321	1,496,1,1	127	520	1,0,208,311	1,518,0,1	127	484	0,0,162,322	0,484,0,0	127
4396	G	A.T.C	3	503	307,194,1,1	501,2,0,0	127	525	283,228,5,9	511,12,2,0	127	492	267,221,1,3	488,2,2,0	127
4723	C	T.A.G	580	499	245,254,0,0	499,0,0,0	127	517	249,265,3,0	514,0,3,0	127	490	235,247,8,0	482,0,8,0	127
5056	C	T.A.G	214	499	259,240,0,0	499,0,0,0	127	534	264,268,1,1	532,0,2,0	127	513	271,239,0,3	510,1,2,0	127
5408	C	T.A.G	329	480	212,265,1,2	477,2,1,0	127	504	246,257,0,1	503,1,0,0	127	509	252,256,1,0	508,0,1,0	127
5475	C	A.G.T	846	507	229,149,83,46	378,129,0,0	127	516	284,167,32,33	451,65,0,0	127	525	294,220,9,2	514,11,0,0	127
6212	T	C.A.G	304	544	331,212,0,1	543,1,0,0	127	538	275,263,0,0	538,0,0,0	127	535	260,275,0,0	535,0,0,0	127
6859	C	A.G.T	194	506	261,242,2,1	503,2,1,0	127	541	250,291,0,0	541,0,0,0	127	552	290,260,0,2	550,1,1,0	127
7100	T	A.C.G	999	460	259,156,25,20	415,44,1,0	127	482	307,151,14,10	458,24,0,0	127	477	90,49,195,143	139,338,0,0	127
7157	T	C.A.G	943	493	264,229,0,0	493,0,0,0	127	519	260,234,10,15	494,25,0,0	127	532	74,79,192,187	153,378,1,0	127
7219	C	T.A.G	999	512	271,232,4,5	503,8,1,0	127	532	234,254,24,20	488,41,2,1	127	511	1,1,217,292	2,507,2,0	127
7242	T	C.A.G	999	497	326,171,0,0	497,0,0,0	127	515	269,211,22,13	480,34,1,0	127	487	78,68,177,164	146,339,2,0	127
7344	A	T.C	999	468	192,275,0,1	467,1,0	127	486	184,256,15,31	440,46,0	127	504	66,90,127,221	156,348,0	127
7357	T	C.A.G	999	514	187,326,0,1	513,0,1,0	127	518	178,292,14,34	470,47,1,0	127	495	62,97,126,210	159,334,1,1	127
7371	G	A.T.C	31	521	137,286,34,64	423,95,1,2	57	515	161,308,17,29	469,45,1,0	127	507	189,317,0,1	506,0,1,0	127
7412	A	G.T	803	517	208,308,0,1	516,0,1	127	532	223,275,16,18	498,34,0	127	513	215,258,18,22	473,40,0	127
7490	A	G.T.C	999	522	276,236,6,4	512,10,0,0	127	521	262,220,26,13	482,38,1,0	127	529	2,4,304,219	6,522,1,0	127
7565	A	G.T.C	999	505	270,196,23,16	466,37,1,1	127	521	268,221,17,15	489,32,0,0	127	522	90,58,207,167	148,372,2,0	127
7674	A	G.T.C	999	516	300,214,0,2	514,0,1,1	127	509	267,208,18,16	475,34,0,0	127	543	24,24,283,212	48,493,1,1	127
7724	A	G.T.C	999	522	314,207,0,1	521,0,1,0	127	528	282,211,21,14	493,33,2,0	127	536	11,10,309,206	21,510,4,1	127
7804	A	T.G.C	999	548	319,228,1,0	547,1,0,0	127	562	287,235,26,14	522,40,0,0	127	576	96,57,275,148	153,423,0,0	127
7842	A	G.T.C	117	550	279,271,0,0	550,0,0,0	127	549	270,258,11,10	528,20,1,0	127	573	320,251,1,1	571,0,2,0	127
7882	T	A.G.C	999	520	237,165,75,43	402,117,1,0	105	512	278,190,32,12	468,44,0,0	127	555	378,175,2,0	553,1,0,1	127
7971	G	A.T.C	215	497	296,185,8,8	481,13,3,0	127	514	259,245,9,1	504,9,1,0	127	555	361,187,3,4	548,1,6,0	127
9514	A	G.T.C	999	531	130,91,189,121	221,309,1,0	127	524	38,13,289,184	51,473,0,0	127	504	296,200,4,4	496,7,1,0	127
9561	C	T.A.G	246	513	356,156,1,0	512,0,1,0	127	546	400,143,2,1	543,0,3,0	127	500	356,141,1,2	497,0,3,0	127
9622	G	A.T.C	459	491	307,181,1,2	488,0,3,0	127	531	330,200,0,1	530,0,1,0	127	486	290,195,1,0	485,0,0,1	127
9921	C	T.A.G	459	526	242,283,1,0	525,0,1,0	127	531	289,241,0,1	530,0,0,1	127	465	245,220,0,0	465,0,0,0	127
10040	A	C.T.G	8	482	236,149,58,39	385,93,2,2	34	543	272,224,29,18	496,47,0,0	127	509	282,225,1,1	507,0,2,0	127
10250	C	T.G.A	999	514	75,69,210,160	144,366,4,0	127	521	17,11,273,220	28,493,0,0	127	469	0,0,259,210	0,466,2,1	127
10313	A	G.T	999	490	80,75,209,126	155,335,0	127	498	187,157,88,66	344,154,0	127	456	47,42,208,159	89,367,0	127
10879	G	T.C.A	628	508	195,309,2,2	504,2,2,0	127	567	229,334,2,2	563,1,2,1	127	463	176,285,1,1	461,1,1,0	127
10940	G	A.T.C	459	580	279,295,4,2	574,4,2,0	127	611	301,305,2,3	606,2,3,0	127	537	306,227,1,3	533,0,4,0	127
11023	G	T.C.A	311	564	283,280,0,1	563,1,0,0	127	597	277,317,3,0	594,1,2,0	127	556	329,225,2,0	554,1,1,0	127
11262	T	C.A.G	999	496	51,34,231,180	85,409,1,1	6	522	200,151,85,86	351,171,0,0	127	511	311,199,1,0	510,1,0,0	127
11291	T	C.A.G	999	504	223,149,80,52	372,132,0,0	127	540	279,215,23,23	494,46,0,0	127	507	297,209,0,1	506,0,1,0	127
11821	A	T.C.G	999	507	187,128,117,75	315,192,0,0	127	540	308,188,30,14	496,43,1,0	127	544	285,183,47,29	468,75,1,0	127
11869	A	G.T.C	999	533	20,10,308,195	30,501,2,0	127	555	238,136,113,68	374,180,1,0	127	542	1,0,358,183	1,540,1,0	127
12166	T	C.A.G	999	522	159,143,108,112	302,220,0,0	127	547	253,261,27,6	514,31,1,1	127	527	1,6,328,192	7,519,1,0	127
12168	T	C.G.A	999	524	157,143,108,116	300,223,0,1	127	548	253,263,26,6	516,31,0,1	127	541	1,8,329,203	9,531,1,0	127
12187	T	C.A.G	999	506	152,111,135,108	263,240,0,3	127	533	285,200,33,15	485,46,2,0	127	557	1,3,341,212	4,551,2,0	127
12193	A	G.C.T	999	536	170,118,142,106	288,247,1,0	127	551	294,210,33,14	504,45,0,2	127	560	2,3,336,219	5,553,0,2	127
12314	T	G.A.C	999	531	145,149,127,110	294,236,0,1	127	566	289,225,27,25	514,49,3,0	127	574	92,76,222,184	168,404,2,0	127
12526	A	G.T.C	999	526	192,180,88,66	372,150,3,1	127	543	218,272,26,27	490,51,1,1	127	498	7,2,236,253	9,485,4,0	127
12618	C	T.A.G	474	498	223,275,0,0	498,0,0,0	127	529	283,245,1,0	528,0,1,0	127	20	11,9,0,0	20,0,0,0	63
12811	A	G.T.C	999	534	19,12,316,187	31,500,3,0	127	540	219,123,124,74	342,196,2,0	127	504	0,0,333,171	0,502,1,1	127
12825	T	A.G.C	999	524	200,154,115,55	354,169,1,0	127	548	303,203,31,11	506,42,0,0	127	488	3,7,319,159	10,478,0,0	127
12836	A	T.G.C	87	383	222,157,2,2	379,4,0,0	127	494	309,185,0,0	494,0,0,0	127	27	4,8,0,15	12,15,0,0	30
12844	G	A.T.C	459	520	264,180,47,29	444,76,0,0	101	536	291,186,35,24	477,57,2,0	127	411	270,130,4,7	400,11,0,0	127
12879	A	G.T.C	999	436	213,195,14,14	408,27,0,1	127	516	281,213,13,9	494,19,3,0	127	208	8,5,153,42	13,194,1,0	127
12903	C	A.T.G	762	432	201,215,7,9	416,9,7,0	127	517	270,236,8,3	506,7,2,2	127	52	10,8,11,23	18,15,19,0	49
13017	G	A.C.T	207	354	150,198,6,0	348,5,0,1	127	510	259,249,1,1	508,0,0,2	127	22	9,5,7,1	14,8,0,0	19
13029	G	T.A.C	999	546	175,170,149,52	345,200,1,0	127	566	284,225,40,17	509,56,0,1	127	490	9,3,269,209	12,476,1,1	127
13085	A	G.T.C	999	496	217,127,129,23	344,150,2,0	56	533	330,156,35,12	486,44,3,0	127	343	9,2,206,126	11,331,1,0	127
13140	T	C.A.G	999	538	178,174,137,49	352,184,2,0	127	563	234,249,52,28	483,79,1,0	3	339	8,1,198,132	9,326,3,1	127
13143	A	G.T.C	151	517	313,190,7,7	503,13,1,0	127	519	281,237,0,1	518,0,1,0	127	336	207,129,0,0	336,0,0,0	127
13165	A	C.T	47	398	165,229,2,2	394,3,1	127	491	217,271,0,3	488,0,3	127	12	5,4,0,3	9,3,0	3
13196	C	T.A.G	999	528	156,231,70,71	387,140,0,1	127	559	213,288,35,23	501,57,1,0	127	493	6,4,286,197	10,483,0,0	127
13254	G	A.T.C	9	362	186,172,3,1	358,3,1,0	127	485	266,217,1,1	483,1,1,0	127	11	8,2,1,0	10,1,0,0	25
13287	T	C.A.G	999	489	20,6,222,241	26,461,2,0	127	518	180,125,102,111	305,213,0,0	127	417	1,0,195,221	1,412,2,2	127
13314	C	T.A.G	531	428	190,148,48,42	338,89,1,0	127	498	223,228,22,25	451,43,4,0	127	173	6,6,86,75	12,160,1,0	67

	GR1.2				GR2.1				IT1.1				LS1.1				LS1.2				LS2.1				LS3.1				YM1.1				B			
	DP	DP4	DPR	GQ	DP	DP4	DPR	GQ	DP	DP4	DPR	GQ	DP	DP4	DPR	GQ	DP	DP4	DPR	GQ	DP	DP4	DPR	GQ	DP	DP4	DPR	GQ	DP	DP4	DPR	GQ	DP	DP4	DPR	GQ